Evolution of reproductive barriers between the two hybridizing sister species *Sepsis cynipsea* and *S. neocynipsea* (Diptera: Sepsidae)

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SUMMARY

The work presented in this dissertation explores the processes of reproductive isolation between the two sister species of black scavenger (or dung) flies *Sepsis cynipsea* and *S. neocynipsea* (Diptera: Sepsidae) and its underlying evolutionary mechanisms. *Sepsis cynipsea* is ubiquitous and most abundant in Europe, whereas *Sepsis neocynipsea* abounds in North America but also occurs at high elevations in Europe, where there is potential for natural hybridisation. Quantitative analysis of the population structure with highly polymorphic, neutral microsatellite and morphological data revealed clear differences between the two species (Chapter 1). Analysis of pre- and postmating isolating barriers preventing hybridisation were the main focus of my studies (Chapters 2 & 3). Evolutionary signals of hybridisation and introgression were then also analysed at the genomic level (Chapter 4).

Chapter 1 explores the genetic population structure among and between species and continents using nine highly polymorphic microsatellite markers as well as morphological data. We took a neutral population genetic null-approach to understand the relative importance of drift vs. sexual and natural selection in producing morphological divergence of a male secondary sexual trait (armored femur of the foreleg) and wings between species and populations of *S. cynipsea* and *S. neocynipsea*. Neutral genetic distances were of similarly high magnitude between but very low within species and continents. Male foreleg morphology showed a clear differentiation between the three lineages following the neutral differentiation but rather being driven by sexual selection. Wing morphology showed a clear phylogenetic signal differentiating the two species most likely driven by stabilizing natural selection.

In **Chapter 2** we quantified the degree of prezygotic isolation and geographic variation in mating behaviour for four populations each of *S. neocynipsea* that occur in allopatry, parapatry, or sympatry with four populations each of its sister species *S. cynipsea* in con- vs. heterospecific crosses, as well as in F_1 hybrid and backcrosses. This study documents successful hybridization under laboratory conditions, with low copulation frequencies in heterospecific pairings but higher frequencies in pairings of F_1 hybrids signifying a possible breakdown of isolating barriers. Longer copulation

latencies in heterospecific pairings suggest some species recognition. Female shaking duration, indicating reluctance to mate and/or female choice, differs strongly between species and appears to contribute to avoiding heterospecific males. Shaking duration was shown to be partially maternal inherited. Females of both species discriminated more strongly against males in areas of sympatry than allopatry. This study highlights an important role of character displacement affecting mating behaviour between hybridizing sepsid species in geographic areas of co-existence.

In **Chapter 3** we quantified the degree of postzygotic isolation with fertility and fecundity measurements with the same approach as in Chapter 2, including the F_2 hybrid generation. Our data revealed strong but not absolute postzygotic barriers between the sister species, with limited indications of intrinsic isolating barriers among continental *S. neocynipsea* populations, signifying they are indeed the same species. As expected, fecundity and fertility were significantly reduced in heterospecific F_1 and F_2 crosses compared to the conspecific parental crosses, presumably due to intrinsic incompatibilities. We also detected hybrid breakdown across several fecundity and fertility traits with considerable difficulties to form hybrid offspring, potentially due to barriers in sperm transfer, difficulties to form a zygote, and/or a decreased survival between the egg and adult stages. These hybridization difficulties were asymmetric in that they most strongly affected the female *cynipsea* – male *neocynipsea* (CN) direction resulting in no offspring production. Viable F_1 hybrid offspring showed male sterility but no suppression of female fertility according to Haldane's rule.

Lastly, in **Chapter 4** we tested for introgression patterns of ancient or recent gene flow in the whole genome between the two species using ABBA-BABA-statistics. We used whole genome sequences (scaffolds) of one iso-female line (representing one individual) from two sympatric Swiss populations (Zürich and Sörenberg) each for *Sepsis cynipsea* and *S. neocynipsea*. Bidirectional introgression between both species in Zürich but not in Sörenberg was evident. Unidirectional but only almost significant introgression from *S. neocynipsea* of Sörenberg into *S. cynipsea* of Zürich and from *S. cynipsea* from Sörenberg into *S. neocynipsea* from Zürich was detected, where the latter pattern was supported by preliminary analysis of pooled population sequences. Work in progress will provide insights into patterns of introgression across

the species natural ranges as well as the role of latitudinal adaptation in shaping genome-wide patterns of genetic variation.

The research presented here highlights the importance of sexual selection in mediating ongoing hybridization and introgression among closely related sympatrically occurring sister species, thus documenting the speciation processes by integrating behavioural, morphological, life history and genomic methods.

ZUSAMMENFASSUNG

Die hier vorliegende Dissertation untersucht die Prozesse reproduktiver Isolation und ihrer zugrundeliegenden evolutionären Mechanismen zwischen zwei Schwesternarten der Schwingfliegen *Sepsis cynipsea* und *S. neocynipsea* (Diptera: Sepsidae). *Sepsis cynipsea* ist omnipräsent in Europa, während *Sepsis neocynipsea* in Nord Amerika häufig, selten aber auch in höheren Lagen Europas vorkommt, wo Potential für natürliche Hybridisierung der beiden Arten gegeben ist. Die quantitative Analyse der Populationsstruktur mit hoch polymorphen, neutralen Mikrosatelliten und morphologischen Daten offenbarte klare Unterschiede zwischen den zwei Arten (Kapitel 1). Die Analyse isolierender Barrieren vor und nach der Verpaarung, die Hybridisierung verhindern könnten, waren Hauptfokus meiner Studien (Kapitel 2 & 3). Evolutionäre Signale der Hybridisierung und Introgression wurden danach auch auf genomischer Ebene analysiert (Kapitel 4).

Kapitel 1 untersuchte die genetische Populationsstruktur innerhalb und zwischen den Arten und Kontinenten mithilfe von neun hoch polymorphen Mikrosatellitenmarkern und morphologischen Daten. Anhand einer neutralen populationsgenetischen Null-Hypothese untersuchten wir die relative Wichtigkeit von genetischer Drift vs. sexueller und natürlicher Selektion, um die morphologische Divergenz eines männlichen sekundären sexuellen Merkmals (gepanzerter Femur des Vorderbeines) und der Flügel zwischen den Arten und Populationen von *S. cynipsea* und *S. neocynipsea* zu erklären. Die Morphologie der männlichen Vorderbeine zeigte eine klare Differenzierung zwischen den drei Erblinien (Arten sowie Kontinente), ähnlich wie bei der neutralen Differenzierung, wobei dieses Muster eher durch sexuelle Selektion hervorgerufen wurde. Die Flügelmorphologie zeigte ein klares phylogenetisches Signal, welches die beiden Arten, nicht jedoch die Kontinente, differenziert und wahrscheinlich durch stabilisierende natürliche Selektion bedingt ist.

In **Kapitel 2** quantifizierten wir den Grad der präzygotischen Isolation und der geographischen Variation im Paarungsverhalten für je vier Populationen von *S. neocynipsea*, die in Allopatrie, Parapatrie bzw. Sympatrie mit je vier Populationen ihrer Schwesternart *S. cynipsea* in Europa und Nordamerika vorkommen. Dabei wurden kon- und heterospezifische parentale Kreuzungen, F_1-Hybrid- sowie

Rückkreuzungen zwischen F_1 und der parentalen Generation untersucht. Diese Studie dokumentiert erfolgreiche Hybridisierung unter Laborbedingungen mit erwartet geringen Kopulationshäufigkeiten bei heterospezifischen Verpaarungen, aber höheren Häufigkeiten bei Verpaarungen zwischen den F_1 Hybriden. Letzteres könnte durch einen möglichen Zusammenbruch isolierender Barrieren hervorgerufen werden. Längere Kopulationslatenzen bei heterospezifischen Verpaarungen deuten auf Arterkennung an. Die Dauer der weiblichen Schüttelphase, welche Verpaarungs-Widerwillen und/oder weibliche Partnerwahl indiziert, unterscheidet sich stark zwischen den Arten und scheint zur Vermeidung heterospezifischer Paarungen beizutragen. Die Schütteldauer ist partiell maternell vererbt. Weibchen beider Arten diskriminierten stärker gegen sympatrische als gegen allopatrische heterospezifische Männchen. Diese Studie dokumentiert die wichtige Rolle von Charakterverschiebung als zentraler Faktor bei der verhaltensbedingten Verhinderung von Verpaarungen zwischen hybridisierenden Sepsidenarten in sympatrischen geographischen Gebieten.

In **Kapitel 3** quantifizierten wir den Grad der postzygotischen Isolation anhand von Fertilitäts- und Fekunditätsmessungen bei den gleichen Populationen, Arten und Generationen wie in Kapitel 2. Unsere Daten zeigten starke, jedoch nicht absolute postkopulatorische Barrieren zwischen den Schwesternarten. Zudem zeigten sich geringe intrinsisch isolierende Barrieren zwischen den kontinentalen Populationen von *S. neocynipsea*, sodass wir zum Schluss kommen können, dass sie in der Tat (noch) der gleichen Art zugehörig sind. Wie erwartet waren Fekundität und Fertilität bei F_1 und F_2 Hybridkreuzungen aufgrund intrinsischer Inkompatibilitäten signifikant reduziert verglichen mit den konspezifischen parentalen Kreuzungen. Es zeigten sich bei mehreren Fekunditäts- und Fertilitätsmerkmalen beachtliche Schwierigkeiten, Hybridnachkommen zu formen, möglicherweise aufgrund von Barrieren während des Spermientransfers, Schwierigkeiten bei der Zygotenbildung und/oder verminderten Überlebenschancen zwischen dem Ei- und dem Adultstadium. Diese Probleme bei der Hybridisierung waren asymmetrisch, wobei die Kreuzungsrichtung Weibchen *cynipsea* – Männchen *neocynipsea* (CN) am stärksten betroffen war, bei der keine Nachkommen produziert wurden. Lebende F_1 Hybridnachkommen zeigten Sterilität des Männchens, jedoch keine Verminderung der weiblichen Fertilität, Haldane's Regel entsprechend.

Abschliessend suchten wir in **Kapitel 4** im gesamten Genom mittels ABBA-BABA-Statistiken nach Introgressionsmustern, die historischen Genfluss zwischen den beiden Arten in der Natur belegen. Wir nutzten ganze Genomsequenzen (Scaffolds) einer isogenischen weiblichen Linie zweier sympatrischer Schweizer Populationen pro Art (Zürich & Sörenberg), welche jeweils ein Individuum repräsentieren. Bidirektionale Introgression zwischen den Arten war nur in Zürich, aber nicht in Sörenberg evident. Wir fanden zudem unidirektionale, jedoch nur fast signifikante Introgression von Sörenberger *S. neocynipsea* aus hinein in Zürcher *S. cynipsea*, wie auch von Sörenberger *S. cynipsea* aus hinein in Zürcher *S. neocynipsea*. Dieser Befund wurde auch durch entsprechende vorläufige Analysen gepoolter Populationssequenzen unterstützt. Weiterführende Arbeiten werden uns Einblicke in die genom-weiten Introgressionsmuster über das gesamte geografische Vorkommen beider Arten hinweg gewähren, inklusive möglicher latitudinaler Klimaanpassungen.

Die hier vorliegende Forschungsarbeit hebt die wichtige Rolle der sexuellen Selektion hervor, die die stetige potentielle Hybridisierung und Introgression zwischen nah verwandten, sympatrisch vorkommenden Schwesternarten formt bzw. verhindert. So dokumentiert diese Dissertation den Artbildungsprozess integrativ mit Methoden der Verhaltensbiologie, der Morphologie, der Lebenszyklusbiologie und der Genomik.

GENERAL INTRODUCTION

The forces underlying speciation are one of the most actively investigated areas of evolutionary biology. The traditional biological species concept only allows for gene flow within species, with absolute reproductive barriers preventing gene exchange between incipient and diverged species (Coyne & Orr, 2004; Dobzhansky, 1951; Mayr, 1963). Many theories were constructed to understand the various modes of speciation explaining the origin of species. Populations can diverge in allopatry, as they will accumulate incompatibilities between alleles in different lineages, if only by chance (genetic drift), leading to strong postzygotic isolation (Dobzhansky, 1936). Mayr (1954) described genetic bottlenecks as an important factor driving speciation, an event drastically reducing effective population sizes and therefore producing rapid speciation afterwards in response to shifting ecological optima of a population, with new adaptation processes afterwards leading to a new lineage (Turelli, Barton, & Coyne, 1999). More recently, researchers focused on incipient species in geographic areas of co-occurrence to understand speciation processes in sympatry driven by disruptive selection and reinforcement (Dieckmann & Doebeli, 1999; Turelli, Barton, & Coyne, 2001).

Historically, speciation was driven by various events. Most prominent is the climatic change of the glacial periods as a bottleneck with massive species extinctions, the consequent formation of refuges, and the re-colonization of those parts of the world after the ice was melted (Hewitt, 1999, 2000). European diversity hotspots were found in the regions south of the Alps or the Pyrenees, such as the Iberian peninsula or Italy, which run east-west and acted as main refuges for numerous species across all taxa (Brito, 2007; Hewitt, 1999). In North America the major mountainous regions, the Rocky Mountains, run north south, and therefore the subsequent differentiation pattern occurred east west (Fedorov & Stenseth, 2012). When studying species diversification these geographic patterns should be considered together with selection and adaptation (Barrowclough *et al.*, 2004; Schmitt & Muller, 2007), as a species' migration history will play a vital role in generating isolation preventing gene flow among species (Coyne & Orr, 2004).

Sexual selection can be much stronger than natural selection and therefore can lead to rapid trait diversification of reproductive morphology even beyond the natural fitness optima (Hosken & House, 2011). Numerous behavioural and morphological

traits contributing to mating are reported to evolve extremely fast in response to sexual selection (Albert, Uy, & Borgia, 2000; Puniamoorthy, Schäfer, & Blanckenhorn, 2012; Rohner, Blanckenhorn, & Puniamoorthy, 2016) than neutral traits (Arnqvist, 1998; Hosken & Stockley, 2004; Puniamoorthy, Su, & Meier, 2008; Eberhard, 2013). Therefore, behavioural and morphological differences in sexual and nonsexual traits between species and populations can help understand how sexual selection acts on phenotype differentiation.

Hybridization as a creative process of speciation

Underlying all the above speciation models are reproductively isolating barriers of different types or modes that are crucial for heterospecific mate recognition as well as conspecific mate preferences (Dobzhansky & Mayr, 1944). Nevertheless, research on diverging populations of widespread incipient sister species to study evolution in action is relatively rare, as most studies focus on already evolved species with geographically often limited distribution (Via, 2001). Speciating populations in sympatry, parapatry, and ultimately also allopatry need to develop isolating mechanisms preventing gene flow, either at the precopulatory, postcopulatory and prezygotic, and/or the postzygotic levels (Coyne & Orr, 2004; Panhuis *et al.*, 2001; Seehausen, van Alphen, & Witte, 1997).

Numerous studies have emphasized the role of allopatric speciation processes for the advent of prezygotic barriers. Female preferences can most easily evolve for any (random) male trait via sexual selection between allopatric populations (Turelli, Barton, & Coyne, 2001). Traditionally, trait and preference were seen as additive polygenic traits with female preferences only evolving after the advent of a male trait (Lande, 1981). More recent studies demonstrated that female preferences can be under independent selection and may evolve as a pleiotropic side effect of alleles selected for different reasons (Price, 1998; Schluter & Price, 1993; Kirkpatrick & Ryan, 1991), ultimately leading to strong prezygotic mating barriers. Almost absolute reproductive barriers can occur via postzygotic isolation when hybrids either show lower fitness in the same environment compared to their parental species (extrinsic postzygotic barriers), or when they show developmental distortions (intrinsic postzygotic barriers; Coyne & Orr, 1998). For sympatric populations speciation mostly arises from adaptation to distinct ecological niches (termed ecological speciation: Schluter, 2000; Via, 2001). Another important factor that is less

intensively researched is reproductive character displacement causing sympatric speciation via reinforcement, a pattern by which heterospecific mate discrimination should be stronger in sympatric than in allopatric populations (Noor, 1999).

All these possible processes, acting alone or in combination, strongly but in some cases presumably not absolutely prevent interbreeding between species; hence, albeit rare, hybridization should be observable regularly and recurrently in nature at any point in time. The resulting first generation (F_1) hybrid offspring are believed to have mostly lowered fitness, which is disadvantageous and therefore should reinforce the speciation processes. Nevertheless, recent studies emphasize the role of introgression in speciation. The traditional model of biological speciation allowed for gene flow only between populations of one species, while heterospecific intercrossing was neglected due to lack of empirical evidence. The evolution of two species from one common ancestor was possible due to a bifurcating process driven by reproductive isolating barriers arising by ecological, spatial, or temporal niche differentiation (Coyne & Orr, 2004; Dobzhansky, 1951; Mayr, 1963, Schluter, 2000). Nevertheless, heterospecific gene flow between diverged species can additionally play a vital role in the speciation process (Fontaine *et al.*, 2015; Payseur & Rieseberg, 2016), as first botanists and later zoologists detected the possibility of introgressive hybridization beyond species boundaries (Anderson, 1949; Gante *et al.*, 2016; Rieseberg *et al.*, 2003; Mallet, 2007). Hybridization was commonly seen primarily as a disadvantage when producing hybrid offspring with low fitness, but it can also help diversification in providing potentially adaptive genetic variation (Seehausen, 2004; Berner & Salzburger, 2015), by providing beneficial introgressed alleles that were already under selection in one of the parental species (Saetre, 2013), or by instantaneously forming a new unit of hybrid species that is reproductively distinct from either of its parental species (Arnold & Meyer, 2006). In geographic areas of co-occurrence, fast and recent species diversification can lead to incomplete lineage sorting and hence to regular hybridization. As selection can act on early hybrids with maladaptive traits, it is important to understand the interaction between selection and recombination in hybridization (Barton & Bengtsson, 1986; Baird, 1995). Analysing such incipient, sympatric species will therefore yield insights into the mechanisms of speciation being affected by introgression.

What to expect in this dissertation

The following four chapters of my dissertation represent an integrative study of the evolution of reproductive isolation between the two widespread sister species *Sepsis cynipsea* and *S. neocynipsea* (Diptera: Sepsidae). *Sepsis cynipsea* is the most common sepsid species in north-central Europe, whereas *Sepsis neocynipsea* abounds in North America, where it essentially takes the niche *S. cynipsea* has in Europe, but it also occurs at high elevation sites in Europe (Pont & Meier, 2002), such as in the Swiss Alps, where there is potential for natural hybridisation. Chapter 1 documents the underlying population genetic differentiation and investigates the role of sexual selection in morphological differentiation of species and populations. Chapters 2 & 3 then investigate pre- and postmating isolation between the species to establish in the laboratory that hybridization indeed can and does happen. The final Chapter 4 then studies historic introgression at the genomic level in areas of co-existence of the two species to ask whether hybridization also occurs in nature.

The chapters are presented as separate manuscripts, with Chapter 2 being accepted in *Animal Behaviour*, and Chapter 1 & 3 soon to be submitted in *Journal of Evolutionary Biology*. Hence some parts are inevitably repetitive.

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CHAPTER ONE

Patterns of genetic differentiation in sexual and neutral morphological traits among species and populations of dung flies (Diptera: Sepsidae)

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ABSTRACT

The relative contribution of adaptive and non-adaptive processes leading to morphological divergence among lineages is of longstanding interest in evolutionary research. The evolution of male specific traits with important functions during mating and fertilization has been of particular interest given their prospective roles during early stages of species formation. Using a neutral population genetic framework obtained from microsatellite analysis we examined geographic patterns of quantitative genetic differentiation of an exaggerated male trait (the armored foreleg femur) and the wings among allopatric and sympatric populations of the closely related black scavenger (or dung) flies *Sepsis neocynipsea* and *S. cynipsea*. Microsatellite analysis revealed clear clustering according to biological species status and substantial differentiation between New and Old world populations of *Sepsis neocynipsea,* but absence of significant population structure within each of the three lineages over large geographic distances. Landmark-based morphometric analysis of eight North American and three European *S. neocynipsea* as well as seven European *S. cynipsea* populations reared at constant 18°C and 24°C in a laboratory common garden revealed significant morphological differentiation in forelegs and wings as well as moderate phenotypic plasticity of the study traits. While patterns of wing shape differentiation followed the traditional species boundaries with no differences between continents in *S. neocynipsea,* suggestive for stabilizing natural selection, male femur shape was split according to continent and species origin similar to patterns of neutral genetic variation. Nevertheless, within lineages we found significant geographic differentiation in femur shape, which did not coincide with neutral expectations. Furthermore, male femur shape showed a strong allometric relationship with body size, as it is often the case for exaggerated male secondary traits, but wing shape much less so. Our findings have important implications for understanding the great diversity and evolutionary dynamics of sex-specific morphologies within the dipterans.

INTRODUCTION

Speciation and geographical variation

To comprehend the immense species richness in the insect clade and animals in general, the evolutionary processes leading to the splitting of lineages need to be understood. Speciation relies on the emergence of reproductive barriers, which can be

induced by a variety of factors. The most obvious way for speciation to proceed is through geographic barriers leading to separate gene pools across a species' range. In a situation of geographic isolation, genetic drift and natural selection can lead to population differentiation and eventually speciation given enough time (Lande, 1980). Evidence for such allopatric speciation is abundant and has been demonstrated for various taxa including birds (Coyne & Price, 2000), ticks (Beati *et al.*, 2013), and amphipods (Stevens & Hogg, 2004). Speciation in sympatry and parapatry are theoretically more intriguing due to the homogenizing effect of gene flow (Slatkin, 1985, 1987). With gene flow reduced to a contact zone (parapatry), selective forces and drift determine the speed of divergence and can outbalance gene flow even over small scales (Turelli, Barton, & Coyne, 2001). As theory and empirical examples have shown, the homogenization through gene flow may also be outpaced in sympatry due to disruptive selection favoring ecologically specialized phenotypes (Barluenga, Stölting, & Salzburger, 2006; Grant, 1999). Assortative mating (Baylis, 1976; Bush, 1966, 1969) and reduced fitness of intermediate phenotypes (Svedin *et al.*, 2008) may then complete the process of speciation.

Sexual selection

Sexual selection on mate and gamete recognition traits is considered a potent force facilitating the evolution of reproductive isolation and speciation (Albert, Uy, & Borgia, 2000; Puniamoorthy, Kotrba, & Meier, 2010; Soto *et al.*, 2013). Due to high variance in mating and fertilization success, sexual selection can be much stronger than natural selection and can lead to rapid diversification of reproductive traits even beyond their natural fitness optima (Hosken & House, 2011). Numerous behavioral (e.g. Albert, Uy, & Borgia, 2000), physiological (Eberhard & Cordero, 1995), and morphological traits such as genital structures, which are cited to diverge much faster than other morphological traits, most likely due to intense sexual selection (Arnqvist, 1998; Eberhard, 2013; Hosken & Stockley, 2004; Puniamoorthy, Su, & Meier, 2008). However, in many cases it is not clear whether sexual selection acts more or less continuously on a given trait as could be expected under certain models of sexual competition and conflict, or whether selection acts only during early stages of speciation to minimize costly interspecies hybridization in geographical areas of co-existence. An animal group in which many questions concerning modes of sexual selection, ecology, and behavior have been addressed, while others regarding

population differentiation or trait evolution remain largely unanswered, is the family of sepsid flies (Diptera: Sepsidae).

The genus Sepsis

The genus *Sepsis* provides an attractive system to investigate the evolutionary forces leading to morphological diversification during different stages of speciation. With about 320 species described, sepsids are a relatively small family of black scavenger or dung flies with a well-resolved phylogeny (Zhao *et al.*, 2013). Many species evolved striking diversity in foreleg morphology, which is frequently used to delineate closely related but otherwise morphologically indistinguishable species, and which appear to have evolved in response to sexual selection (Ang, Puniamoorthy, & Meier, 2008; Blanckenhorn *et al.*, 2004; Dmitriew & Blanckenhorn, 2012; Eberhard, 2002; Puniamoorthy, Su, & Meier, 2008; Puniamoorthy *et al.*, 2009). Male flies use their strongly modified femur to hold on to the female's wing base while mating. Whether this trait aids in species recognition, prevents other males from taking over the mate, is part of female choice through quality assessment, or even stimulates sensory cells at the wing base to induce copulation is still discussed. The premating behavior strongly varies in type and intensity across the phylogeny, including diverse elements of courtship (Eberhard, 2013; Puniamoorthy, Su, & Meier, 2008; Puniamoorthy *et al.*, 2009), female choice and resistance (Blanckenhorn *et al.*, 2000; Puniamoorthy, Blanckenhorn, & Schäfer, 2012), and male-male competition (Rohner, Blanckenhorn, & Puniamoorthy, 2016; Ward, 1983; Ward, Hemmi, & Rösli, 1992).

Despite extensive research on sexual selection in this group, no studies have tried to assess the relative importance of different evolutionary forces for speciation in this genus. The sister species *S. cynipsea* and *S. neocynipsea* are still able to hybridize, but intermediate genotypes have reduced fertility (Giesen, Blanckenhorn, & Schäfer, 2017, Chapters 2, 3). Whilst *S. neocynipsea* occurs in North American as well as Europe, *S. cynipsea* is restricted to Eurasia (Ozerov, 2005; Pont & Meier, 2002). The factual exclusion of gene flow between continental ranges of *S. neocynipsea* makes it an outstanding system to explore the role sexual selection leading to morphological divergence in geographic areas in allopatry and sympatry. In this study, we first analyzed the population and phylogenetic structure between the three lineages of European *S. cynipsea*, European as well as North American *S. neocynipsea* with nine highly polymorphic, neutral microsatellite markers. We further

used common garden laboratory rearing of flies from multiple populations of all three lineages at two temperatures (18°C, 24°C) to compare geographic differentiation patterns of quantitative traits (the armored foreleg femur and wing morphology) with the null expectation based on the neutral microsatellite markers. Male wing morphology might only be slightly affected by sexual selection, as this trait is not directly involved in mating in these two species, but may be naturally selected (Klepsatel *et al.*, 2014), whereas male foreleg morphology is obviously sexually selected (Ingram *et al.*, 2008). Our results provide new insights into the evolutionary dynamics and processes contributing to morphological divergence of a secondary sexual trait at different stages of speciation.

MATERIAL & METHODS

Study organism

The sister species *S. cynipsea* and *S. neocynipsea* (Diptera: Sepsidae) exhibit only little differentiation of the mitochondrial barcoding genes COI and CytB (Su, Kutty, & Meier, 2008). Moreover, the species show differentiation in morphology, behavior, and ecology (Pont & Meier, 2002; Puniamoorthy *et al.*, 2009). *Sepsis cynipsea* is the most abundant sepsid in north-central Europe, where it occurs in sympatry with the rare *S. neocynipsea* in some mountainous regions such as the Swiss Alps. In contrast, *S. neocynipsea* occupies the same warm-adapted temperature niche in North America, where *S. cynipsea* is absent (Pont & Meier, 2002). The mating system of *S. cynipsea* has been described in detail (Blanckenhorn, 1999; Blanckenhorn *et al.*, 2000; Parker, 1972a, b; Puniamoorthy *et al.*, 2009; Ward, 1983; Ward, Hemmi, & Rösli, 1992), while only little is known about *S. neocynipsea* (Eberhard, 1999; Puniamoorthy *et al.,* 2009; Rohner, Blanckenhorn, & Puniamoorthy, 2016). A detailed description of the maintenance of the flies in our laboratory and an ethical note is given in Giesen, Blanckenhorn, & Schäfer (2017, Chapter 2).

Microsatellite genotyping and data analyses

A total of 338 specimens from 17 European *S. cynipsea* populations, 116 specimens from 12 US-American and 108 specimens from 6 European *S. neocynipsea* populations were collected to represent the distributional range of both species on two continents (Appendix Table A1; Fig. 1). Genomic DNA was isolated from whole flies using DNeasy Blood and Tissue kit (Qiagen AG, Hombrechtikon, Switzerland)

according to the manufacturer's protocol. Genotyping was done for nine highly polymorphic microsatellite markers following the M13-tail PCR method (Schuelke, 2000). Six of these markers were already isolated for *S. cynipsea* (Greminger *et al.*, 2009); we additionally designed three more markers (J60, G53, E67) for amplification in both species (see Appendix Table A3). We followed the protocol for PCR amplification and separation as described in detail in Greminger *et al.* (2009). Appendix Table A4 provides more information on the sample sizes, the number of alleles, mean observed (H_O) and expected (H_E) heterozygosity for each locus across North American and European populations.

To explore and illustrate the genotypic data, we build up a Neighbour Joining (NJ) tree using the package *poppr* in R (R Development Core Team, 2015) based on the proportion of shared allele distance matrix for populations calculated with the *memgene* package in R. Node support was calculated among 10'000 bootstrap replicates resampled using *poppr*. Quantification of the degree of genetic differentiation resulting from separation among continents relative to that originating from the differentiation between populations within continents was performed with the ade4 AMOVA implementation in the *poppr* package. We further performed a Mantel test (Manly, 1991) with the program ZT (Bonnet & van de Peer, 2002) comparing matrices of pairwise F_{ST}-values with matrices of pairwise geographical distances. Pairwise and global F_{ST}-values were estimated according Weir & Cockerham (1984), while statistical significance was determined by permuting genotypes among populations 10000 times. These calculations were carried out with Microsatellite Analyzer Version 3.12 (Dieringer & Schlötterer, 2003).

Common garden rearing for subsequent morphometric analyses

For the comparative analysis of male foreleg and wing morphology, offspring from a total of 228 iso-female lines of *S. cynipsea* and *S. neocynipsea* were collected at various places and kept for variable times (i.e. number of generations) in our laboratory. *S. cynipsea* originated from seven distinct locations in central and northern Europe. North American *S. neocynipsea* originated from eight populations collected in the United States of America and Canada. For comparison, three European *S. neocynipsea* populations from the Swiss Alps, where they co-occur with *S. cynipsea*, were additionally examined (Appendix Table A2; Fig. 1). All flies were raised in a common garden environment at two temperatures (18°C, 24°C) and controlled

density at unlimited food conditions (sugar, water, and cow dung) for one generation. After emergence, adult flies were stored in 70% EtOH at -18°C and, for each line and temperature regime, three randomly chosen males were used for morphometric analysis.

Morphometric data acquisition and statistical analysis

Forelegs and wings were removed from the thorax in 70% EtOH and, after evaporation of the ethanol, embedded in Euparal (Carl Roth GmbH, Karlsruhe, Germany) on a microscopy slide in always the same orientation to minimize potential errors arising from orientational variation. Slides were then placed on a 50°C heating plate for five minutes to liquefy the resin before samples were dried at room temperature.

Morphometric analyses were performed using landmarks describing shape variation extracted from digital photographs made with a LeicaDFC490 mounted on a Leica MZ12 microscope. Seven landmarks were placed to describe shape variation of the male foreleg femur, marking distinct and most probably interspecifically homologous points. In addition, three sliding, evenly spaced semi-landmarks (Gunz & Mitteroecker, 2013) were placed between landmarks One and Two as well as landmarks Six and Seven to measure the curvature of the leg between the fixed landmarks (Fig. 3a). Sixteen landmarks were chosen for the wings, marking all vein-node positions in the center of the wing and at the wing margin (Fig. 3d).

Landmarks were acquired using *tpsutil* Version 1.21.0.1 (Rohlf, 2015) and *tpsdig2* version 1.1 (Rohlf, 2006). Centroid sizes and full procrustes transformation providing a new set of coordinates for subsequent morphometric analyses as described in detail in Rohlf & Slice (1990) were extracted with *Past* (Hammer, Harper, & Ryan, 2001). A PCA using the procrustes coordinates was produced using the R package *geomorph*, which allows graphical illustration of relative shape changes of landmarks (Adams & Otarola-Castillo, 2013; Klingenberg & Zaklan, 2000; R Development Core Team, 2015).

We used two different approaches to estimate the correlation of shape with size. First, both X and Y coordinates were regressed separately on centroid size. The slope was then used to predict the strength and direction of the shape change of each landmark relative to a given change in size (Mitteroecker *et al.*, 2013), yielding a graphical illustration of the complete shape change relative to a size change, which

were graphically illustrated with R packages *geomorph* and *ggplot2* (Wickham, 2009). The second approach correlated each principal component (PC) with centroid size using linear regression to facilitate the interpretation of different shape components in a population genetic and sexual selection framework.

Geographic patterns of morphological differentiation across species and populations were analyzed by performing nested linear mixed effect models on the different PCs and the centroid sizes, with iso-females nested within populations and population within the three lineages (i) European *S. cynipsea*, (ii) North American, and (iii) European *S. neocynipsea*. Temperature and lineage were treated as fixed factors, population and iso-female line as random factors. The interaction with temperature was included at all levels. Partial η^2 was used to estimate effect size.

RESULTS

Phylogeographic patterns of microsatellite variation

A Neigbour Joining (NJ) tree based on the proportion of shared alleles illustrates that populations of *S. neocynipsea* and *S. cynipsea* clearly cluster according to species status (Fig. 2). The NJ tree further shows that North American and European populations of *S. neocynipsea* form distinct and well-supported clades. Branch lengths within lineages were relatively short indicating that populations share a large proportion of alleles. Merely the *S. neocynipsea* population from Zurich showed some degree of genetic distinctness relative to the other three populations collected from the Swiss Alps. This finding is not surprising and coincides with the distribution of *S. neocynipsea* in central Europe, where the species is abundant at high altitudes but extremely rare in lowland habitats.

Similar results were obtained from analyses of microsatellite differentiation. AMOVA revealed that 20.1% of the total genetic variance is explained by the differences among the three lineages, and only 0.9% could be attributed to differences among populations within lineages. The remaining 78.5% of the total molecular variance was localized within populations. Further pairwise comparisons indicated strong genetic differentiation between *S. cynipsea* and North American populations of *S. neocynipsea* (F_{ST} = 0.22; $p < 0.001$), whereas the corresponding differentiation within Europe was somewhat lower (F_{ST} = 0.16; $p < 0.001$) and of similar magnitude as that between New and Old world populations of *S. neocynipsea* (F_{ST} = 0.16; $p < 0.001$).

The degree of genetic differentiation among populations within each of the three lineages was very low but nevertheless statistically significant (*S. cynipsea*: F_{ST}= 0.01, p < 0.001; *S. neocynipsea* Europe: F_{ST} = 0.01, p = 0.001; *S. neocynipsea* North America: F_{ST} = 0.03, p < 0.001). Mantel tests further yielded a significant correlation between pairwise F_{ST}-values and spherical geographic distances across Europe in *S. cynipsea* (r = 0.45, p = 0.011), but this correlation largely depended on the Estonian population from Pehka, which was significantly differentiated from all other populations. When this population was excluded from the analysis the Mantel correlation turned non-significant (r = 0.19, p = 0.13). No pattern of isolation-by-distance was evident across North American populations of *S. neocynipsea* (r = -0.15, p = 0.77). Due to low sample size we did not test for isolation-by-distance among European *S. neocynipsea* populations.

Shape descriptors of forelegs and wings

PCs accounting for less than 10% of the total morphological variation were omitted from any further analysis, since they contain mostly random variation and are difficult to interpret given the overwhelming shifts explained by PCs accounting for higher proportions of variation.

The two major PCs cumulatively explained 71.39% of the total shape variation in male femur morphology. PC1 accounted for 51.03% of the shape variation and is primarily related to the width of the femur along the dorso-ventral axis. Flies with negative PC1 scores show wider femurs relative to flies with positive scores (Fig. 3b). PC2 explained 20.36% of the shape variation and describes predominantly the depth of the notch (LM 4) and the relative positioning of the main setae at the ventral side of the femur (LM 5 & LM 6). Lower scores of PC2 represent a more protruding attachment of the first main seta and a flatter shaped notch (Fig. 3c).

Three PCs accounted for 69.83% of the total shape variation in wing morphology. PC1 explained 43.42% of the variation largely describing the shape of the wing margin. The ratio of wing length to wing width, called wing aspect ratio, is frequently used to describe the overall shape. In the present study, high PC1 scores correspond to a low wing aspect ratio. In addition, more elongated wings (high wing aspect ratio) are associated with a shift of the anterior and posterior cross-veins towards the base of the wing, while more roundish wings tend to have both central

cross-veins more distally located (Fig.3e). PC2, accounting for 13.43% of the total variation, characterizes a convergence of the anterior (LM 13 & LM 14) and posterior cross-veins (LM 15 & LM 16). Furthermore, flies with high PC2 value have wider wings at the 3rd posterior cell, caused by a more proximal positioning of the 5th longitudinal vein (LM 16 & LM 7) and a slight shift of the anterior wing margin (Fig. 3f). PC3, which explains 12.98%, largely describes wing width. Compared to PC2, which shifts LM 7 and LM 16 in proximal direction, PC3 is associated with a shift in posterior direction. Furthermore, high PC3 values are related to a shift of the anterior cross-vein towards the base of the wing, while low values displace it to a more distal position (Fig. 3g).

Allometric relationships of foreleg and wing shape

Overall, large femurs were much wider (i.e. rounder) than small femurs. The allometric slope was of similar magnitude in North American (Fig. 6a, blue) and European populations of *S. neocynipsea* (Fig. 6a, green), and slightly weaker in *S. cynipsea* (Fig. 6a, black). The three lineages further revealed differences in the relative positioning of the attachment of the main setae at the ventral side of the femur, which strongly co-varied with centroid size in North American and European populations of *S. neocynipsea* but to a much lesser extent in *S. cynipsea*. Additionally, the notch (LM 4 in Fig. 3a) indicated a strong x-directional shift in the European *S. neocynipsea* lineage. Linear regressions of the different PCs on centroid size yielded a similar picture (Table 1a; Fig. 6a). PC1 strongly correlated (negatively) with femur size in all lineages. On average 70% of the variation of *S. neocynipsea* in PC1 can be explained by centroid size, while in the *S. cynipsea* lineage only 40% of the variation is attributable to femur size. PC2 also significantly correlated with size in all lineages, most strongly so in the European *S. neocynipsea* lineage (not shown).

In contrast to femur shape, the allometric components of the PCs extracted for wing shape were less pronounced, albeit also statistically significant (Table 1b; Fig. 6b). In general, larger wings tended to be more roundish with the anterior and posterior cross-veins situated more centrally in the wing in all lineages (Fig. 4 b,d,f). However, *S. neocynipsea* featured more size dependent variation in respect to the 3rd posterior cell (compare Fig. 4b with 4d,f), mostly caused by a shift of the end of the anal cross-vein (LM 8 in Fig. 3d). Similar to femur morphology, PC1 depended significantly (positively) on size in all lineages, although the relationship was

relatively weak (Fig. 6b). The proportion of variation explained by size was higher in *S. neocynipsea* than *S. cynipsea,* and within *S. neocynipsea* considerably higher in European than American populations. The relationship is close to isometry in all lineages (see slopes in Table 1b) and mostly caused by the relative shortening of the wing in larger individuals and an increase in size of the 3rd posterior cell relative to other wing cells. PC2 only indicated a significant (isometric) relationship with wing size in American flies. The analysis of PC3 again showed significant allometric relationships in all lineages, which were however very weak (Table 1b, not shown).

Geographic patterns of morphological differentiation

Nested linear mixed effect models revealed significant quantitative genetic differentiation in male foreleg morphology between and within lineages (Table 2a). In agreement with earlier population-based studies of body size variation (Rohner, Blanckenhorn, & Puniamoorthy, 2016), North American populations of *S. neocynipsea* had larger femurs than European *S. neocynipsea* and *S. cynipsea*. As illustrated in Fig. 5a*,* PC2 clearly separates the two species *S. cynipsea* and *S. neocynipsea*, while PC1 separates North American *S. neocynipsea* from the other two lineages. Significant morphological differentiation was also evident within continents, as population varied significantly for PC1, PC2 and centroid size (Table 2a).

For the wings, PC1 showed a strong phylogenetic signal clearly supporting the taxonomic distinction of the two species. *S. cynipsea* was found to have more elongated wings (as indicated by negative PC1 scores) than *S. neocynipsea,* which evolved more roundish wings (as indicated by positive scores of PC1; Fig. 5b; compare Figs. 4b and 4d,f),). PC2 was only marginally differentiated among the three lineages, and PC3 and centroid size did not indicate any lineage differentiation (Fig. 5b; Table 2b).

Similar results were obtained patterns within lineages, where populations were geographically differentiated in foreleg but only weakly in wing shape (Fig. 5; Table 2). Iso-female line effects, as main effect or in interaction with temperature, were strong throughout, indicating substantial standing genetic variance encoding for foreleg and wing morphology.

Temperature-dependent plasticity

Two temperature regimes were applied in the common garden experiment to address

phenotypic plasticity of the investigated traits. Contrary to expectations, temperature effects were in general quite weak and statistically non-significant with regard to the PCs analyzed for the male femur and the wings (Table 2). Nevertheless, temperature influenced femur size but not wing, size such that flies raised at 18°C developed larger femurs compared to flies at 24°C. In addition, we found significant temperature by population interactions affecting centroid sizes of foreleg and wing size, suggesting a genetic basis of phenotypic plasticity.

DISCUSSION

Our analysis of geographic patterns of genetic differentiation in morphological and molecular variation in two closely related sepsid fly species from two continents yielded four main results. First, microsatellite analyses showed clear phylogenetic differentiation under neutrality between European *S. cynipsea*, European *S. neocynipsea*, and North American *S. neocynipsea*, with at the same time very little differentiation within the three lineages. Second, morphometric analyses revealed stronger divergence in male foreleg than wing morphology among species and populations. Third, wing shape differentiation followed the traditional biological species concept with pronounced differentiation between the species but no significant differentiation between the continents in *S. neocynipsea*. Lastly, forelegs were differentiated between the continents (in PC1) as much as between the species (in PC2: Fig. 5) similar to the results for the microsatellites, and additionally showing stronger allometry between shape and size. In the following we first discuss the phylogeny of the three lineages, and then we consider the potential role of adaptive and non-adaptive evolutionary processes that might have contributed to trait diversification of forelegs and wings.

Phylogeographic and demographic history

Phylogeographic molecular studies provide insights into the evolutionary history of the studied species, and reveal present day patterns of gene flow and drift. So far, only the differentiation between the two species *S. cynipsea* and *S. neocynipsea* has been resolved with barcording genes COI and CytB as well as morphological data indicating that they are very closely related (Pont & Meier, 2002; Puniamoorthy *et al.*, 2009; Su, Kutty, & Meier, 2008), however leaving the species phylogeographic and demographic history unresolved. Puniamoorthy *et al.* (2013) showed that continental

(European and North American) lineages of *S. punctum* are clearly genetically differentiated, with a haplotype network recovering three geographic clusters in North America, southern, and north-central Europe. As *S. punctum* is related phylogenetically and ecologically to our study species, we also expected two European clusters for *S. cynipsea* north and south of the Alps, as well as continental differentiation for *S. neocynipsea*. The latter was found, the former not (Fig. 2).

The very low microsatellite differentiation among populations of either species likely relates to the generally large effective population sizes of *Sepsis spp.* minimizing genetic drift effects; nevertheless, it was somewhat surprising given samples were collected over wide geographic distances for both European *S. cynipsea* and North American *S. neocynipsea*. Our phylogeny (Fig. 2) confirms that the two species indeed differ, and additionally shows clear differentiation between the continents for *S. neocynipsea* similar in extent to that between European *S. cynipsea* and *S. neocynipsea*. An isolation-by-distance pattern was only evident for European *S. cynipsea*, which however strongly depended on the Estonian population (Fig. 1). Nonetheless, as other populations were equally distant, the Estonian population either faces restricted gene flow or signifies a different glacial refuge. For European populations of the much larger yellow dung fly *Scathophaga stercoraria* Demont *et al.* (2008) argued that western Scandinavian flies represent a distinct lineage that re-colonized Europe along a separate route after the Pleistocene glaciation. Regardless, routes of post-glacial (re-)colonization of cold-adapted species, which often survived in multiple refuges further towards the poles, are in general difficult to detect (Bhagwat & Willis, 2008; Hewitt, 2004), especially in species with good dispersal capacity.

Sexual selection and patterns of morphological divergence

Sexual selection is considered to be a main driving force behind the evolution of exaggerated male secondary traits, here exemplified by the male fore femur (Darwin, 1871; Lande, 1980; Andersson, 1994). Comparative studies of sepsid flies indicate great variation in mating systems implying variable intensities of sexual selection acting on specific male traits in different species. For instance, Puniamoorthy *et. al.* (2012) showed, that a shift in the mating system of *S. punctum* is associated with a continental reversal of sexual size dimorphism (see also Dmitriew & Blanckenhorn, 2012). In European populations characterized by resource defense polygyny and male

aggression, males are larger than females, while in American populations, in which female choice of courting males is more important, males are smaller than females. American and European populations of *S. neocynipsea* present a similar, albeit reversed situation (Rohner, Blanckenhorn, & Puniamoorthy, 2016). Moreover, in *S. cynipsea* large males, have a clear mating advantage relating to their ability to hold on in case of female reluctance to mate (Blanckenhorn *et al.*, 2000), for which their (for allometric reasons) large, extended fore femora should be functionally important (Fig. 3). Thus, its seems plausible that sexual selection at least to some extent contributed to patterns quantitative genetic differentiation in male foreleg morphology among and within lineages. Indeed, laboratory experiments with *S. cynipsea* and *S. neocynipsea* not only revealed significant sexual selection on male femur shape within populations, but also diversifying selection between *S. cynipsea* and European *S neocynipsea* suggestive for character displacement in geographic areas of co-existence (Baur, 2016).

In contrast to the fore femur, no significant geographic differentiation within lineages was evident for wing shape, despite populations harboring significant standing genetic variation indicating that this trait can diverge if exposed to natural selection. Our analysis further indicated that *S. cynipsea* was clearly differentiated from its sister species *S. neocynipsea*, whereas American and European *S. neocynipsea* wings did not differ morphologically (Fig. 5b) despite strong molecular differentiation at neutrally evolving microsatellite loci. Similar conclusions were reached in clinal studies of *Drosophila melanogaster* (Gilchrist *et al*., 2000; Gilchrist & Partridge, 2001) demonstrating weak morphological differentiation in wing shape among populations collected from different continents. The authors discussed that contrary to wing (i.e. body size) wing shape may be phenotypically and genetically more canalized and subject to stabilizing (rather than directional) selection. If stabilizing natural (i.e. viability) selection is responsible for the patterns of quantitative genetic differentiation seen in our flies as documented in birds (e.g. Bumpus, 1899), could not be addressed here.

Trait-size and condition-dependency of femur and wing morphology

Male femur shape (particularly PC1: Fig. 6a) strongly correlates with trait size within all studied lineages. Bonduriansky (2007) argued that male sexual traits are typically highly condition-dependent due to resource-allocation trade-offs (also Bonduriansky

& Day, 2003). Dmitriew & Blanckenhorn (2014) demonstrated strong condition-dependence of body size and mid-leg length in the closely related *S. punctum* in response to food (i.e. dung) quantity manipulations. Our common garden experiment here further revealed that flies raised at lower temperature developed larger fore femurs in accordance with the temperature-size-rule, which applies for almost all ectotherms (Atkinson, 1994). In contrast, no temperature effect on wing size or shape was found, suggesting that femur morphology is more plastic and wing morphology more canalized, as expected for male secondary sexual traits with important function in male-competition and female choice (Eberhard, 2002; Bonduriansky, 2007).

CONCLUSION

This integrative study combined molecular differentiation and morphometric analyses of the two closely related, widespread species *S. cynipsea* and *S. neocynipsea* to understand the evolutionary forces driving their trait divergence. We documented the population genetic differentiation between European *S. cynipsea*, European, and North American *S. neocynipsea* under neutral assumptions, revealing a distinct phylogenetic pattern separating species and the continents. Wing morphology followed the traditional species boundaries with no continental differentiation in *S. neocynipsea* maybe driven under stabilizing natural selection, while the male armored foreleg, a secondary sexual trait, was differentiated between species and continents similar to the neutral pattern but rather being influenced by sexual. We identified sexual selection (on the fore femora) acting as an important force in shaping male secondary sexual traits. Further phylogeographic analyses might indicate character displacement as a potential force acting on shape aspects of male fore leg morphology.

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Fig. 1. Map of the *S. cynipsea* (black) and *S. neocynipsea* from North America (blue) or Europe (green) populations sampled for the microsatellite analysis (light triangles) and the morphological differentiation study (dark circle) in North America (top) and Europe (bottom).

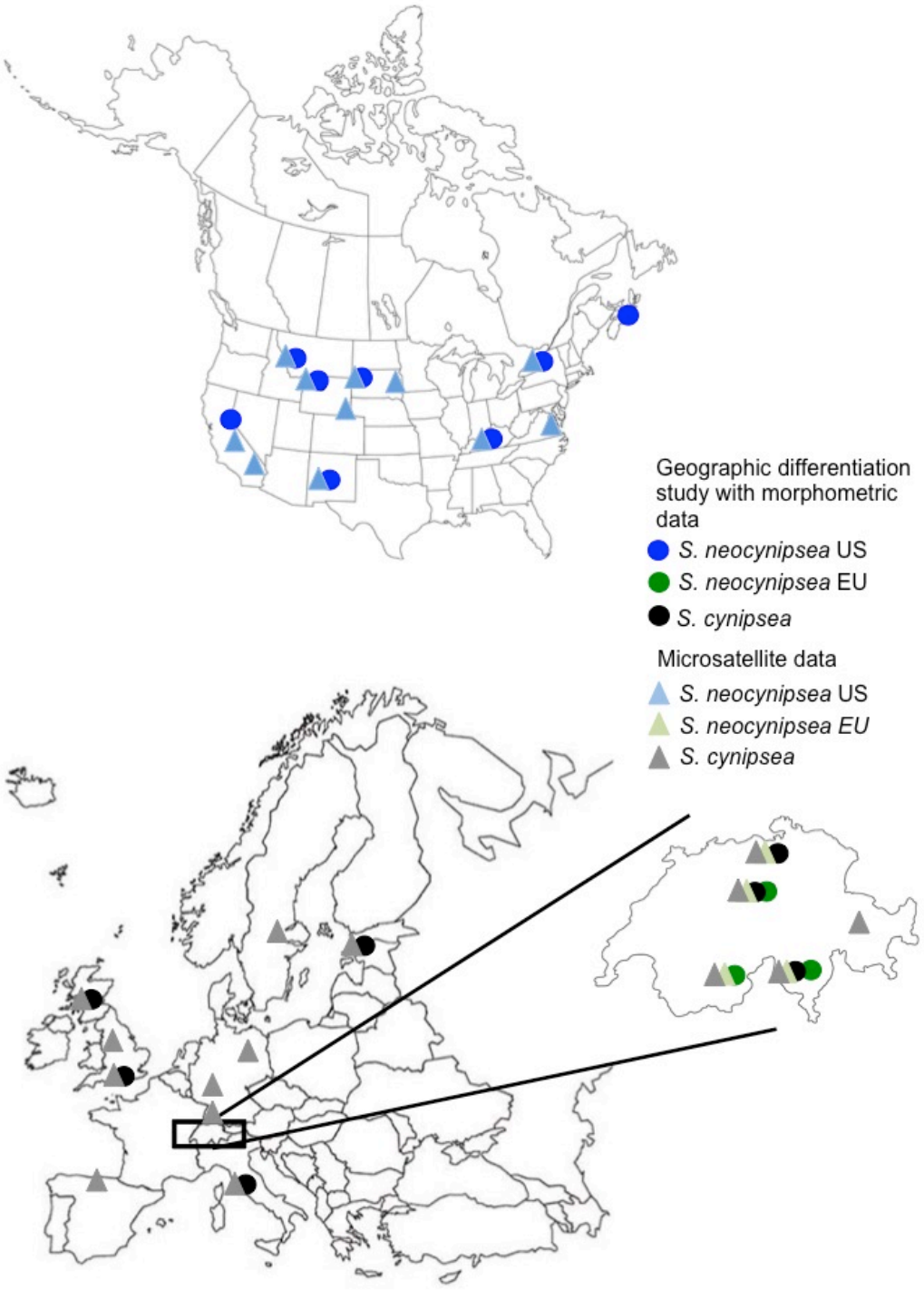

Fig. 2. Neighbor Joining (NJ) tree based on nine highly polymorphic, neutral microsatellite markers for multiple populations of (i) European *S. cynipsea* as well as (ii) European and (iii) North American *S. neocynipsea*.

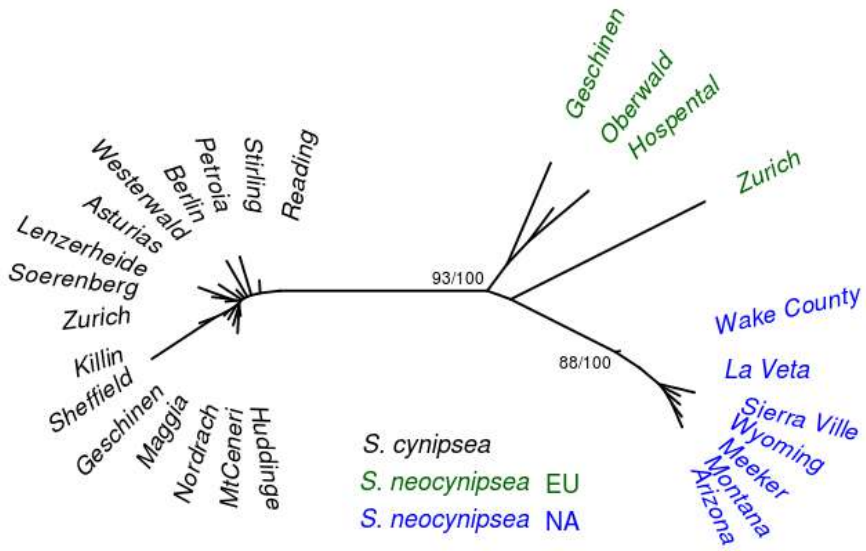

Fig. 3. Morphometric analyses done with a) 13 landmarks for the male fore femur and d) 16 landmarks for the male wing. Shape change for the femur (b, c) and wing (e, f, g) described by PC1 (b, e), PC 2 (c, f), and PC3 (g) illustrate the mean shape (black) as well as the individual with the lowest (red) and highest (blue) score.

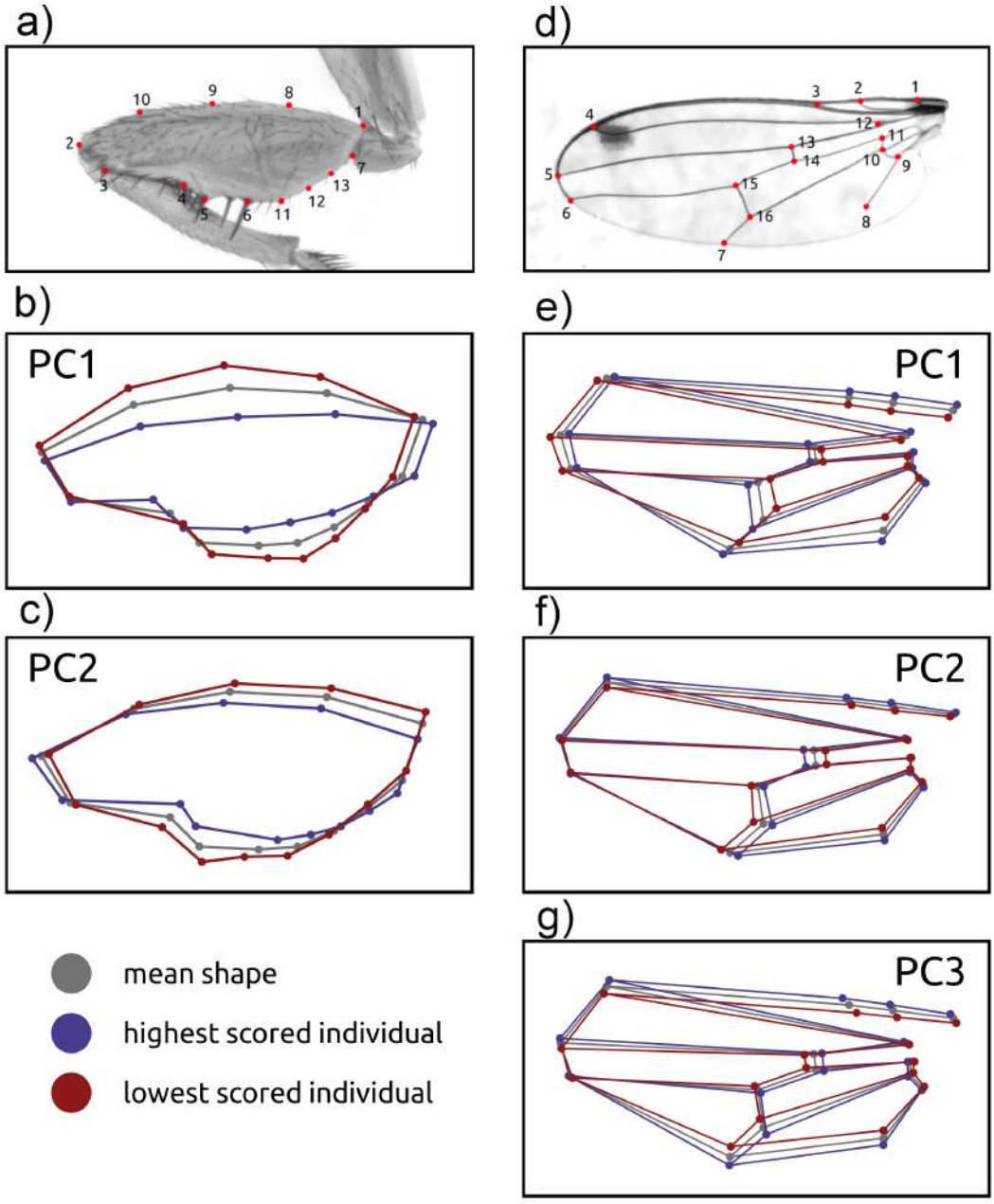

Fig. 4. Size-dependent shape change for *S. cynipsea* (a, b), European (c, d) and North American *S. neocynipsea* (e, f) for femur (a, c, e) and wing (b, d, f).

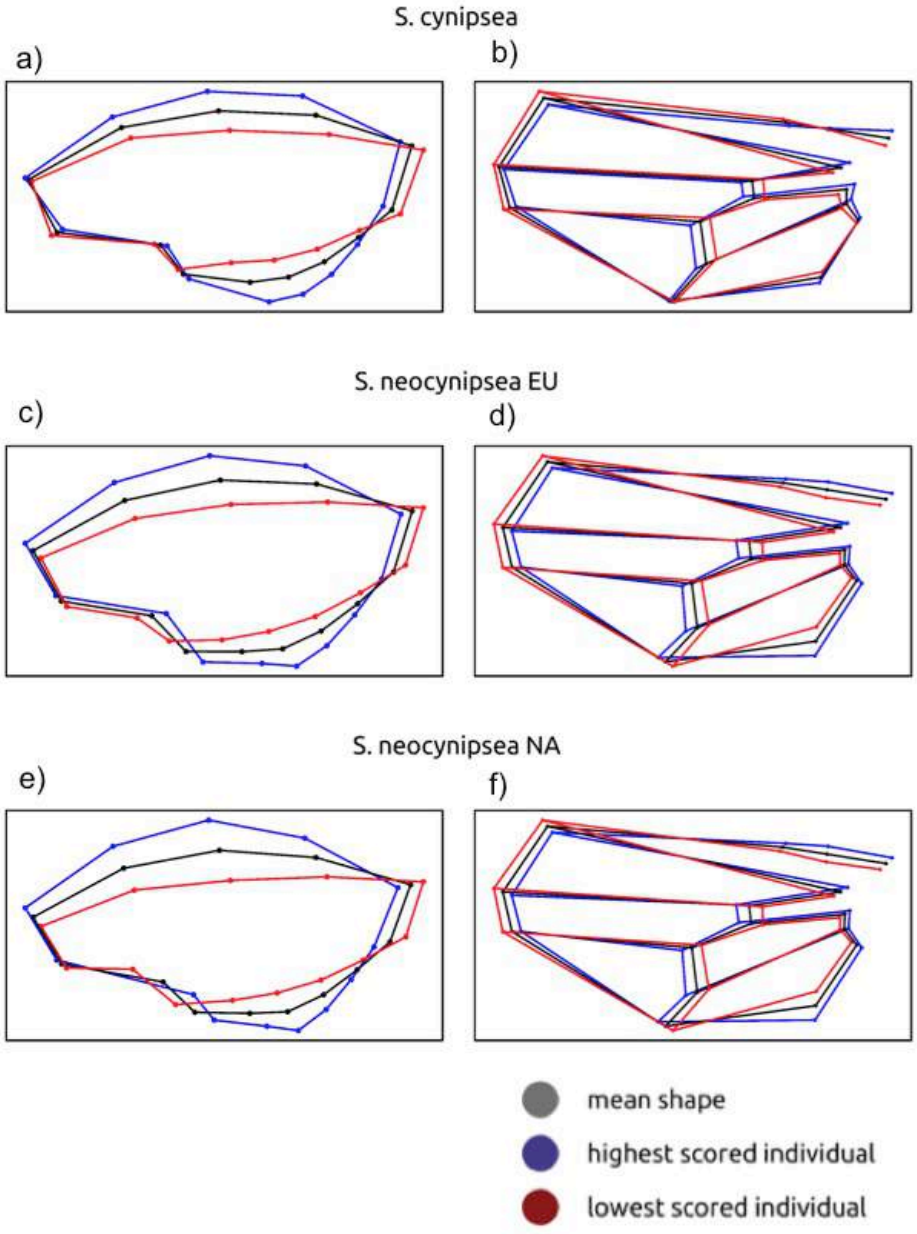

Fig. 5. Morphological differentiation of the male a) fore femur and b) wing in PC1 and PC2 (incl. 95% ellipses) for three lineages and population means (solid dots).

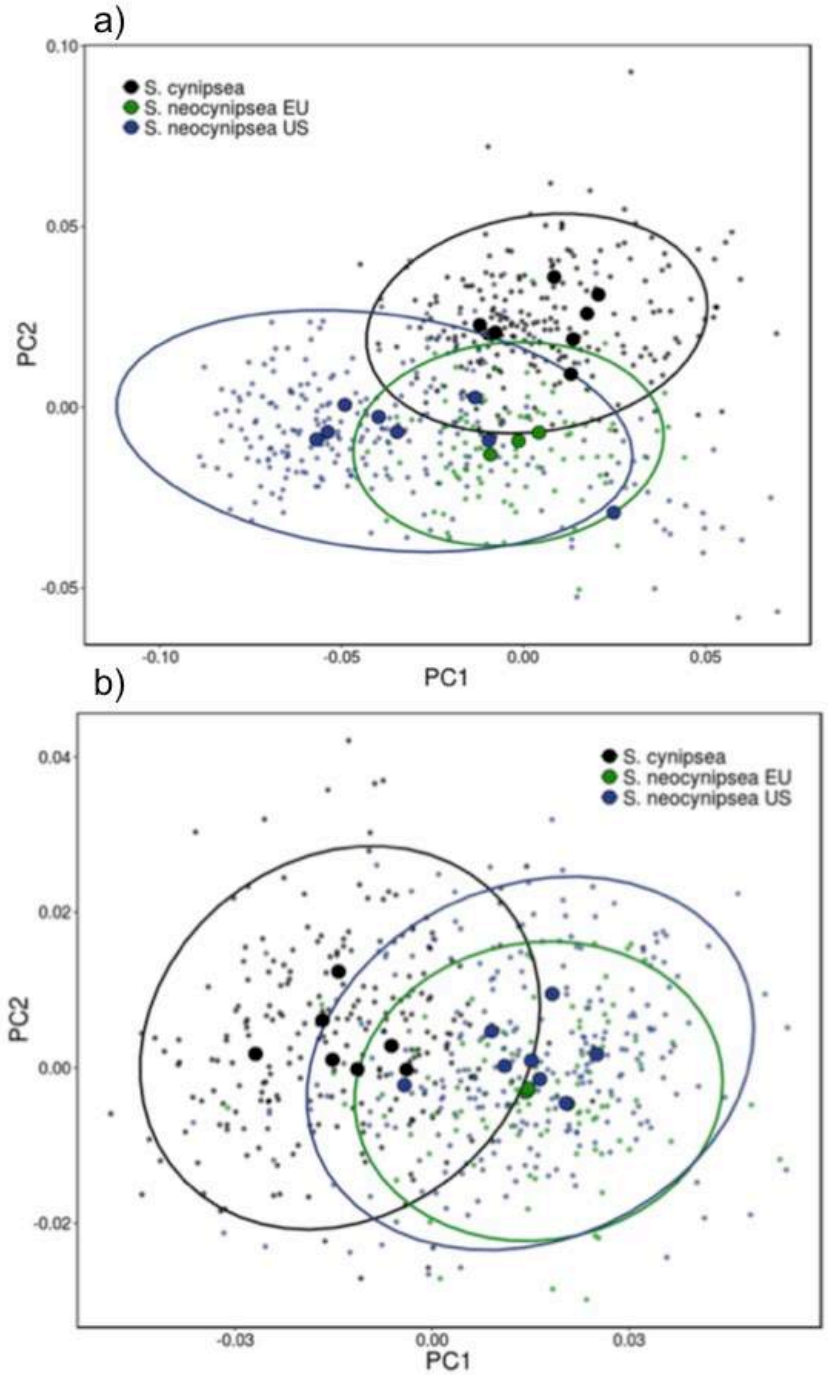

Fig. 6. Allometries of PC1 with centroid size for the male a) fore femur and b) wing.

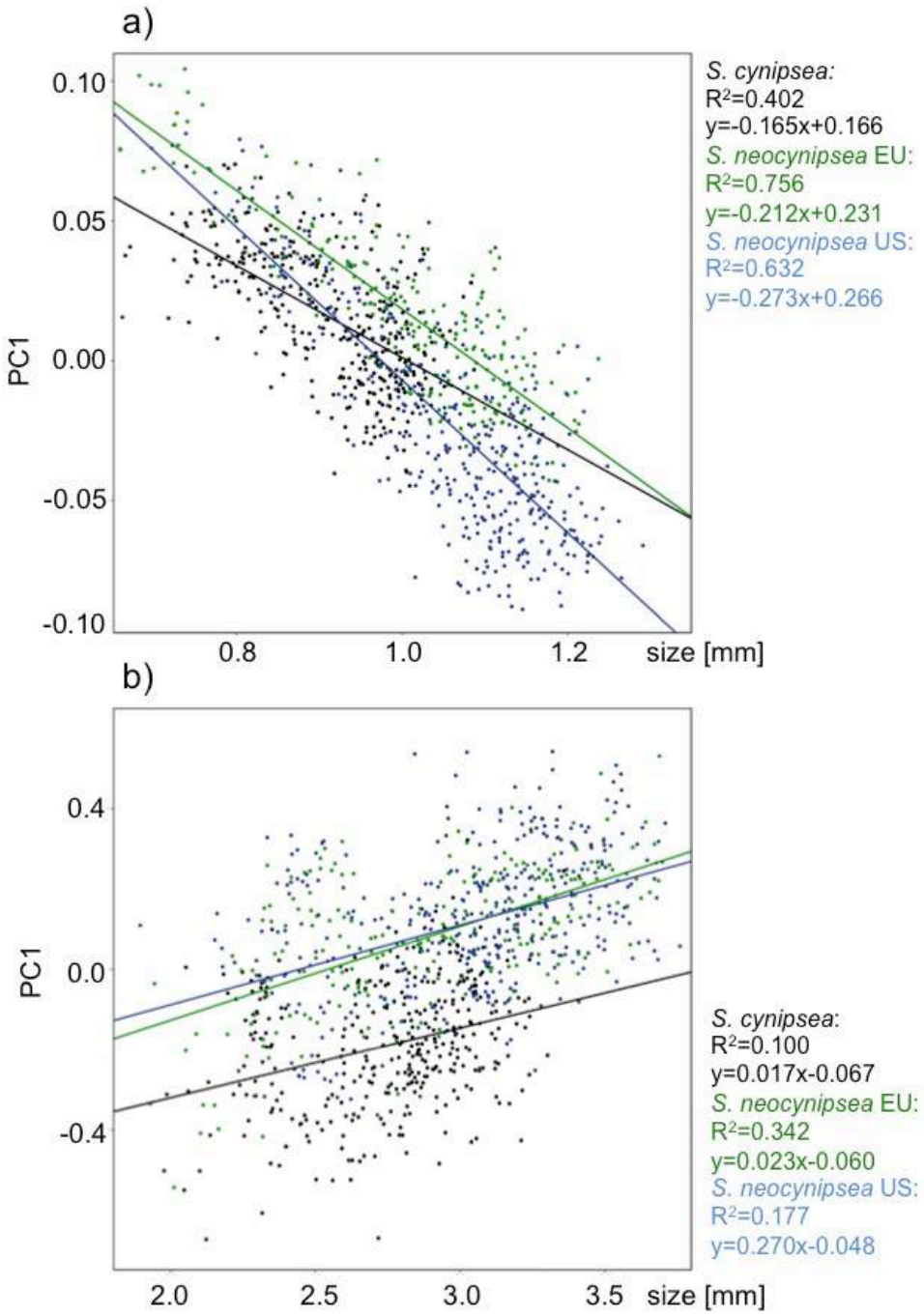

Table 1. Regression of male a) fore femur shape and b) wing-shape components PC1 and PC2 for all three lineages.

a) fore femur shape components

	PC component	R^2	slope	*p*
(i) *S. cynipsea*	PC1	0.40	-4.28	**<0.01**
	PC2	0.12	-2.26	**<0.01**
(ii) European *S. neocynipsea*	PC1	0.76	-5.53	**<0.01**
	PC2	0.37	-3.27	**<0.01**
(iii) North American *S. neocynipsea*	PC1	0.63	-7.11	**<0.01**
	PC2	0.18	-2.65	**<0.01**

b) wing-shape components

	PC component	R^2	slope	*p*
(i) *S. cynipsea*	PC1	0.10	0.79	**<0.01**
	PC2	0.00	-0.07	0.69
	PC3	0.06	0.94	**<0.01**
(ii) European *S. neocynipsea*	PC1	0.34	1.06	**<0.01**
	PC2	0.01	-0.22	0.10
	PC3	0.14	0.88	**<0.01**
(iii) North American *S. neocynipsea*	PC1	0.18	0.90	**<0.01**
	PC2	0.12	-0.96	**<0.01**
	PC3	0.02	0.30	0.01

Table 2. Nested linear mixed effects model for the male a) fore femur and b) wings.

a) model for fore femur

		PC1			PC2			Centroid Size		
	df	F	*p*	η^2	F	*p*	η^2	F	*p*	η^2
L	1	7.20	**0.01**	0.49	38.10	**<0.01**	0.84	17.10	**<0.01**	0.70
T	2	3.23	0.09	0.17	2.43	0.14	0.13	5.04	**0.04**	0.25
L*T	2	0.42	0.67	0.05	1.80	0.20	0.19	1.82	0.20	0.20
Population(L)	15	7.39	**<0.01**	0.84	3.55	**0.01**	0.76	3.48	**<0.01**	0.69
Li(P(L))	122	1.77	**<0.01**	0.74	1.48	**0.03**	0.71	2.48	**<0.01**	0.80
P(L)*T	14	1.84	0.05	0.28	1.58	0.11	0.25	3.87	**<0.01**	0.45
Li(P(L))*T	70	1.77	**<0.01**	0.26	1.65	**<0.01**	0.25	1.61	**<0.01**	0.24
Error	353									

L: Lineage; T: Temperature; P: Population; Li: Iso-female line.

b) model for wing

		PC1			PC2			PC3			Centroid Size		
	df	F	*p*	η^2	F	*p*	η^2	F	*p*	η^2	F	*p*	η^2
L	1	31.38	**<0.01**	0.81	3.76	0.05	0.35	1.29	0.30	0.15	1.72	0.21	0.11
T	2	1.46	0.24	0.09	1.29	0.27	0.07	1.98	0.18	0.12	2.61	0.11	0.26
L*T	2	0.13	0.88	0.02	2.44	0.12	0.24	0.33	0.72	0.04	1.48	0.26	0.17
P(L)	15	2.71	**0.01**	0.58	1.40	0.21	0.40	1.65	0.15	0.57	2.72	**0.03**	0.73
Li(P(L))	120	2.26	**<0.01**	0.78	2.29	**<0.01**	0.78	1.91	**<0.01**	0.75	2.08	**<0.01**	0.77
P(L)*T	14	1.64	0.09	0.25	1.49	0.14	0.24	4.46	**<0.01**	0.48	22.25	**<0.01**	0.82
Li(P(L))*T	70	1.60	**<0.01**	0.24	1.22	0.13	0.19	1.44	**0.02**	0.22	1.97	**<0.01**	0.28
Error	362												

L: Lineage; T: Temperature; P: Population; Li: Iso-female line.

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APPENDIX

Appendix Table A1. Sampling locations and sample sizes of the *S. cynipsea* and *S. neocynipsea* used for the microsatellite analysis.

Species	Location	GPS coordinates	$N_{individuals}$
S. cynipsea	Pehka, EST	59.487, 26.348	15
	Sheffield, UK	53.383, -1.468	18
	Geschinen, CH	46.495, 8.280	25
	Lenzerheide, CH	46.727, 9.556	25
	Asturias, ESP	43.477, -5.891	16
	Dillenburg, GER	50.743, 8.294	25
	Stirling, UK	56.119, -3.932	23
	Killin, UK	56.469, -4.320	12
	Maggia, CH	46.266, 8.685	16
	Monte Ceneri, CH	46.113, 8.934	14
	Reading, UK	51.478, -1.009	17
	Nordrach, GER	48.424, 8.114	25
	Sörenberg, CH	46.823, 8.032	25
	Zürich, CH	47.381, 8.604	25
	Petroia, I	43.233, 12.566	18
	Huddinge, SWE	59.244, 17.945	25
	Berlin, GER	52.483, 13.167	14
S. neocynipsea	Hospental, CH	46.603, 8.582	25
	Oberwald, CH	46.571, 8.368	24
	Sörenberg, CH	46.823, 8.032	20
	Maggia, CH	46.266, 8.685	8
	Zürich, CH	47.381, 8.604	6
	Geschinen, CH	46.495, 8.280	14
	Fort Hall, ID	43.013, -112.454	15
	Lexington, KY	38.102, -84.554	20
	Lamar Valley, WY	44.868, -110.175	11
	Meeker, CO	40.051, -107.902	18
	La Veta, CA	33.722, -117.670	15
	Tucson, AZ	32.209, -111.070	7
	Belgrade, MT	45.793, -111.173	7
	Sierraville, CA	39.583, -120.353	8
	Syracuse, NY	43.016, -76.107	4
	Sheridan, WY	44.773, -106.988	7
	Raleigh, NC	35.870, -78.758	4

Appendix Table A2. Sampling locations and sample sizes of the populations used in the study assessing morphological differentiation.

Species	Continent	Population	Latitude	Longitude	Temperature	# Lines	# Individuals
S. neocynipsea	America	Lamar Valley, WY	44.60	-110.50	18	8	20
					24	8	20
		Charlottetown, PEI	46.23	-63.13	18	4	20
					24	4	20
		Lexington, KY	38.04	-84.50	18	10	20
					24	11	20
		Zephyr Cove, NV	39.00	-119.57	18	6	20
					24	8	20
		Syracuse, NY	42.94	-76.90	18	7	20
					24	5	20
		Belgrade, MT	45.47	-111.11	18	4	10
					24	4	10
		Tucson, AZ	32.13	-110.55	18	5	9
					24	9	18
		Sheridan, WY	44.48	-106.58	18	3	9
	Europe	Sörenberg, CH	46.87	8.27	18	10	20
					24	10	20
		Maggia, CH	46.25	8.70	18	6	12
					24	6	12

Table A2. ff.

		Hospental, CH	46.53	8.35	18	5	18
					24	7	20
S. cynipsea	Europe	Sörenberg, CH	46.87	8.27	18	10	20
					24	10	20
		Maggia, CH	46.25	8.70	18	5	11
					24	9	16
		Zürich, CH	47.22	8.32	18	3	20
					24	3	12
		Killin, UK	56.11	-3.90	18	7	20
					24	6	20
		Reading, UK	51.27	-0.58	18	5	20
					24	4	20
		Pehka, EST	59.48	26.37	18	6	14
					24	7	12
		Petroia, I	43.21	12.34	18	6	14
					24	7	14

Appendix Table A3. Characterization of isolated microsatellite markers cross-amplifying in *Sepsis cynipsea* and *S. neocynipsea*.

Locus Name	Primer Sequence (5'-3')	T_a	allelic size range
J60	F: TGT AAA ACG ACG GCC AGT CGG AAA GTT ACC R: GTT TCT TCG TCG GGA GAG ATA ACA CGA	60°C	160-190
G53	F: TGT AAA ACG ACG GCC AGT GGA GAT GCG TGA CTT R: GTT TCT TGC AAA CGT AAT GCC GAA GAT	60°C	350-420
E67	F: TGT AAA ACG ACG GCC AGT CCG CAG CAG AAC ATC AAC ACA AAT R: TCC GCT TCG AAT CCC GTC AG	60°C	155-197

Appendix Table A4. Analysis of microsatellite markers.

	Europe												North America					
	S. cynipsea (N_{pop} = 17)						*S. neocynipsea* (N_{pop} = 6)						*S. neocynipsea* (N_{pop} = 11)					
Mircosatellite marker	N_{ind}	N_a	H_o	H_e	F_{ST}	p	N_{ind}	N_a	H_o	H_e	F_{ST}	p	N_{ind}	Na	Ho	He	Fst	p
K55	337	7	0.651	0.657	0.008	0.101	97	4	0.052	0.081	0.020	0.149	115	3	0.035	0.043	-0.032	0.940
K11	331	16	0.088	0.862	0.014	<0.001	96	12	0.073	0.838	-0.011	0.741	114	6	0.079	0.239	-0.031	0.863
J60	328	18	0.454	0.891	0.020	<0.001	92	14	0.663	0.749	0.015	0.120	115	11	0.765	0.782	0.070	<0.001
H94	335	19	0.397	0.781	0.012	0.056	96	22	0.688	0.891	0.049	<0.001	115	6	0.435	0.547	0.006	0.355
H26	337	13	0.605	0.634	0.009	0.043	97	11	0.680	0.790	0.026	0.027	115	10	0.548	0.678	0.067	<0.001
G67	331	24	0.801	0.920	0.013	<0.001	96	13	0.833	0.895	0.003	0.360	114	11	0.566	0.829	0.018	0.108
G53	327	74	0.801	0.975	0.004	0.016	81	37	0.630	0.954	-0.005	0.768	112	33	0.580	0.950	0.040	<0.001
E81N	311	11	0.241	0.810	0.025	0.002	96	12	0.490	0.872	0.027	0.016	113	11	0.455	0.667	0.006	0.355
E67	332	12	0.214	0.303	-0.010	0.905	96	6	0.375	0.465	0.002	0.384	113	19	0.717	0.900	0.032	0.002
overall	338						97						116					

N_{pop}: population sample size, N_{ind}: individual sample size, N_a: average number of alleles over all loci; H_o: observed heterozygosity; H_e: expected heterozygosity. In total we sampled 551 individuals of both species and both continents. Genomic DNA was isolated from whole flies using DNeasy Blood & Tissue Kit (Qiagen AG, Hombrechtikon, CH) following manufacturer's protocol. PCR amplification for nine microsatellite markers was done using the M13-tail PCR method (Schuelke, 2000) and is described in detail in Greminger et al. (2009). Amplifications were conducted with 15 min initial denaturation at 95°C, 35 cycles of 30s denaturation at 94°C, 45s annealing cycle at 60°C (except for H94 with 56°C and H26 with 54°C), and 45s at 72°C, followed by 8 cycles of 30s at 94°C, 30s at 53°C, 45s at 72°C, and finally ended with a final extension of 30 min at 60°C. Fluorescent-labeled PCR fragments were separated on an ABI Prism 3730 capillary sequencer and allele lengths were scored using GeneMapper V 4.0 (both Applied Biosystems, Carlsbad, CA).

CHAPTER TWO

Behavioural mechanisms of reproductive isolation between two hybridising dung fly species (*Sepsis cynipsea* and *S. neocynipsea*; Diptera: Sepsidae)

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ABSTRACT

Characterization of the phenotypic differentiation and genetic basis of traits that can contribute to reproductive isolation is an important avenue to understand the mechanisms of speciation. We quantified the degree of prezygotic isolation and geographic variation in mating behaviour among four populations of *Sepsis neocynipsea* that occur in allopatry, parapatry, or sympatry with four populations of its sister species *S. cynipsea*. To obtain insights into the quantitative genetic basis and the role of selection against hybrid phenotypes we also investigated mating behaviour of F_1 hybrid offspring and corresponding backcrosses with the parental populations. Our study documents successful hybridisation under laboratory conditions, with low copulation frequencies in heterospecific pairings but higher frequencies in pairings of F_1 hybrids signifying hybrid vigour. Analyses of F_1 offspring and their parental backcrosses provided little evidence for sexual selection against hybrids. Longer copulation latencies in heterospecific pairings indicate species recognition, probably due to surface or volatile chemicals. The frequency of male mating attempts did not differ greatly among species or hybrid pairings, suggesting no male discrimination of mating partners. Female shaking duration, signifying female choice and/or reluctance to mate, differs strongly between the species and appears to contribute to avoiding heterospecific males; this trait is partially maternal inherited. Importantly, females of both species discriminated more strongly against males in areas of sympatry than allopatry indicating reinforcement. Shorter copulations in heterospecific parental pairings and longer copulations in F_1 hybrids suggest mechanistic difficulties with sperm transfer. Overall, our study highlights an important role of character displacement affecting mating behaviour of hybridising sepsid species in geographic areas of co-existence.

INTRODUCTION

Speciation proceeds gradually from restricted levels of gene flow at early stages to complete reproductive isolation at later stages (Coyne & Orr, 2004; Dobzhansky, 1951; Mayr, 1942). In many cases ecological, spatial or temporal niche differentiation prevents interbreeding between hybridising species (Schluter, 2000, 2001). More interestingly, reproductive isolation may evolve through sexual selection leading to divergence in mate or gamete recognition systems (Kozak, Reisland, & Boughmann, 2009; Svensson *et al.*, 2007; Via, 2001). While theoretical studies have established

sexual selection as an important potential agent in driving the evolution of reproductive isolation (Gavrilets, 2000; Lande, 1981; Turelli, Barton, & Coyne, 2001), supporting empirical data remain scarce and largely restricted to phylogenetic species comparisons over long evolutionary timescales (Panhuis *et al.*, 2001; Kraaijeveld, Kraaijeveld-Smit, & Maan, 2010). As a consequence for many taxa it is unclear whether sexual selection alone causes reproductive isolation independent of species composition within habitats, or whether it acts in a more punctuated manner as predicted for reproductive character displacement in geographic areas of co-existence (Lande, 1981; Gavrilets, 2000; Turelli, Barton & Coyne, 2001). In this context, several authors recently emphasized the necessity to better understand the relationship between micro-evolutionary mechanisms causing trait divergence and macro-evolutionary patterns among lineages showing some degree of reproductive isolation.

Behavioural, morphological (i.e. mechanical) or olfactory differences between incipient species can lead to strong prezygotic isolating barriers, which however may remain incomplete. The main and therefore the strongest barriers result from postzygotic isolation with reinforcement, fertilization problems, and hybrid male sterility (Hood, Egan, & Feder, 2012; Reed & Markow, 2004; Wassermann & Koepfer, 1977). Although reproductive isolation involves many different types of traits, behaviour is considered as a main driving force behind the evolution of reproductive barriers to gene flow (Gleason & Ritchie, 1998; Puniamoorthy *et al*., 2009; Shaw & Herlihy, 2000). For example, Puniamoorthy (2014) demonstrated for the neotropical fly *Archisepsis diversiformis* that qualitatively different courtship behaviours contributed to reproductive isolation between two geographically separated populations otherwise presenting only minor morphological and molecular differentiation.

The closely related sister species *Sepsis cynipsea* and *S. neocynipsea* (Diptera: Sepsidae) offer great opportunity to investigate behavioural mechanisms and underlying evolutionary forces leading to reproductive isolation at early stages of speciation (Via, 2009). Based on their partially sympatric distribution in the Swiss Alps and strong similarities in morphology and behaviour we suspected that these two species might hybridise in nature. We here examine typical mating traits in con- vs. heterospecific parental pairings, F_1 hybrids and backcrosses between Swiss sympatric,

European parapatric, and North American allopatric populations, focusing on behavioural traits common to both species: male mating attempts by jumping on a partner, female shaking during pairing, here likely indicating male assessment, as well as copulation frequency, latency, and duration (Blanckenhorn *et al.*, 2000; Parker, 1972a,b; Ward, 1983). Although the reluctance and assessment functions of female shaking can be hard to distinguish in practice (Blanckenhorn *et al.*, 2000), we expected more pronounced female mate choice in heterospecific pairings following male assessment and species recognition, eventually resulting in reluctance to mate. We further expected lowest hybridisation rates and strongest (i.e. reinforced) behavioural differentiation in the European sympatric populations of the Swiss Alps, and some differentiation between European and North American *S. neocynipsea* due to their spatial separation.

MATERIALS & METHODS

Study organism

Sepsis cynipsea and *S. neocynipsea* (Diptera: Sepsidae) are two closely related species that exhibit clear morphological and behavioural differences (Pont & Meier, 2002) but limited variation in gene sequence data indicating differentiation (Su, Kutty, & Meier, 2008; Puniamoorthy, Su, & Meier, 2008; Baur *et al.*, 2017, Chapter 1). *Sepsis cynipsea* is the most common sepsid species in north-central Europe, while populations of *S. neocynipsea* are present in Europe only in the Alps and other mountainous regions, whereas in North America they abound also at low altitudes, there occupying the ecological niche of the absent *S. cynipsea* (Pont & Meier, 2002). Both similarly breed in fresh cowpats and are reproductively active from spring to late autumn (Eberhard, 1999; Parker, 1972a,b; Pont & Meier, 2002). While the mating system of *S. cynipsea* is well studied (Blanckenhorn *et al.*, 1999, 2002; Hosken *et al.*, 2003; Parker 1972a,b; Puniamoorthy *et al.*, 2009; Rohner, Blanckenhorn & Puniamoorthy, 2016; Ward, 1983; Ward, Hemi, & Rösli, 1992), only little is known about its sister species *S. neocynipsea* (Eberhard, 1999; Puniamoorthy *et al.*, 2009; Rohner, Blanckenhorn, & Puniamoorthy, 2016).

Ethical Note and Maintenance of Flies

No legal regulations for scientific laboratory work with sepsid flies exist in Switzerland, the EU, or the USA such that we did not apply for any licenses or

permits. After 30+ years of experience working with dung flies, our ability to handle them carefully and appropriately is given. We caught wild individuals by swiping a butterfly net over fresh cowpats. Sepsid flies were extracted from the net using an aspirator and transferred into 1L transparent plastic containers with fixed Eppendorf tubes offering sugar and water *ad libitum*. Most other non-target insects so collected were released again on site. Collected live flies were brought or sent to our laboratory, where they were identified by sex and species according to differences in male armoured foreleg morphology. Male flies were stored as voucher specimens in 100% ethanol at -20°C, and gravid females were isolated into round 50 mL glass vials including a rectangular plastic dish (4.2 x 2.1 x 1.6 cm^3) filled with fresh cow dung as oviposition substrate and some grains of sugar. Emerging F_1 offspring of single females were then transferred into 1 x 1 x 1.4 dm^3 plastic containers with fresh cow dung, water *ad libitum*, and sugar for continuous propagation in the laboratory. Iso-female lines were subsequently held in these containers in a climate chamber at 24°C, 60% humidity, and 16h:8h light:dark cycle, with regular maintenance by supplying fresh cow dung every 14 days (rearing conditions detailed in Puniamoorthy, Schäfer, & Blanckenhorn, 2012). We identified species in iso-female lines according their male F_1 offspring. Our experimental flies were derived from these iso-female lines that had been housed and propagated for up to 2 years before our experiment (see Rohner, Blanckenhorn and Puniamoorthy, 2016, for more details). After experiments we froze all flies in 100% ethanol at -20°C.

Fly origin and pairing scheme

Wild caught gravid females were collected from six sites (i.e. populations) to ultimately establish 5 to 15 iso-female lines per population in the laboratory (Table 1). *Sepsis cynipsea* and *S. neocynipsea* were obtained from two areas in Switzerland where the two species co-occur sympatrically (Zurich, Sörenberg). *S. cynipsea* were further collected from two other adjoining European regions, where *S. neocynipsea* has not been observed (Ludwigshafen, Germany, and Stirling, Scotland). However, the literature documents neighboring *S. neocynipsea* appearances in both regions (Pont & Meier, 2002; Ozerov, 2005), so that we classified these populations as parapatric. The other *S. neocynipsea* originated from two allopatric North American populations where *S. cynipsea* does not exist (Fort Hall, Idaho, and Lamar Valley, Wyoming).

With these flies we thus could form reciprocal heterospecific parental pairings of three biogeographical types with two population replicates each: European sympatry, European parapatry, and cross-continental allopatry (Table 2). In parallel, we performed conspecific parental pairings within each of the four populations per species as the baseline for comparison, as well as two reciprocal population replicates of European with North American *S. neocynipsea* as conspecific allopatric cross-continental pairings (Table 2). In all cases, one population replicate consisted of 15 to 20 pairing replicates derived from our iso-female lines. Potentially lower sample sizes in mating experiments with F_1 hybrid offspring were expected due to difficulties in obtaining hybrids. For backcrosses we targeted a sample size of six replicates per pairing, as we set up two reciprocal types (female hybrid with male parental – F_1xP, and female parental with male hybrid – PxF_1) to detect possible sex specific effects. In the end we conducted observations for (1) con- and hetero-specific parental (P) pairings (mean sample size = 19.13, range 15 to 20), (2) F_1 hybrid (F_1) pairings using the offspring resulting from heterospecific pairings (mean sample size = 11.44, range between 3 to 20), and (3) backcrosses (BC) of F_1 hybrid offspring with the parental species (mean sample size = 4.23, range 1 to 6). All pairings were done reciprocally.
Hybrid flies for our behavioural assessments of the F_1 and backcrosses were generated by randomly combining up to 30 flies of one sex from various iso-female lines of a given population and species with a roughly equal number of flies of the other sex from various iso-female lines of a given population of the other species (Table 2; again done reciprocally). Matings in this setting were necessarily heterospecific, and females were allowed to oviposit eggs into fresh cow dung to generate F_1 hybrid offspring for our experiments.

Assessment of Mating Behaviour

For each pairing replicate (cf. above) we combined five virgin females with five virgin male individuals (i.e. 5f:5m) into a round 50 mL (length 8 cm x diameter 2.5 cm) glass vial containing a smear of cow dung, all independently and randomly chosen from the various iso-female lines of a given population. This implies that some of the individuals in each replicate vial may have stemmed from the same iso-female line by chance. Virginity was guaranteed by separating flies by sex within 24 h after emergence. Flies were always aged 3 to 6 days after adult eclosion to ensure sexual maturity (Teuschl & Blanckenhorn, 2007). Due to errors, losses, deaths, or

accidental surplus of individuals, effective group sizes varied between 3f:3m and 6f:6m. We thus followed Puniamoorthy (2014), who reported for *Archisepsis diversiformis* (Diptera: Sepsidae) that hybrids between different Sepsid species were not produced when flies were confronted with only one mating partner of the other population/species, but only when confronting groups of flies, thus emulating the natural situation at cowpats and increasing interaction probabilities (Eberhard, 1999; Parker, 1972a,b).

Observation of mating behaviour started right after fly introduction and lasted for 30 min. We recorded (i) the total number of male mating attempts as an indicator of male willingness to mate, i.e. jumps onto a female, (ii) the cumulative female shaking duration with a mounted male indicating mate assessment and/or reluctance to mate [recorded in seconds, in the Figures converted to min], and (iii) the average duration of all copulations [in min] (Blanckenhorn *et al.*, 2000; Boake, Price, & Andreadis, 1998; Ding & Blanckenhorn, 2002). We always scored the entire copulation duration, even if it exceeded the 30 min observation interval. From these assays we further derived, for final analysis, (iv) the time to first copulation (i.e. copulation latency) as an indicator of how fast mating ensues, and (v) the number of copulations realized per male mating attempt (copulation frequency). After the experiments all flies were frozen and stored in 100% ethanol at -20°C for any future work.

Statistical Analyses

We ultimately standardized all trait measurements (except copulation duration) for analysis to 30 minutes and one pair. The number of male mating attempts, female shaking duration, copulation duration, and latency were $\log_{10}$-transformed for a better residual distribution in parametric statistical tests. Mating frequency was arcsine-transformed for analysis (logistic analyses with binomial errors yielded qualitatively similar results). All five traits were analysed separately, with and without the other traits as covariates because male and female behaviours interact to produce matings (only significant covariates are reported in the Results), with univariate GLMs in SPSS Statistics Version 23. For the parental pairings, a given behavioural trait was analysed as a function of species (*S. cynipsea:* C, *S. neocynipsea*: N, and CxN vs. NxC – female always named first), and biogeographic type nested within species (sympatry vs. parapatry in Europe vs. allopatry across continents) as fixed factors, and population nested within biogeographic type within species as random effect.

Particular pairings were additionally compared (planned comparisons): baseline behaviour of C vs. N; cross-continental vs. within-population N; and direction of heterospecific mating, i.e. CxN vs. NxC. F_1 hybrid and backcrosses were analysed analogously but separately. We also performed two corresponding multivariate analyses subsuming, on the one hand, copulation frequency, male mating attempts, and female shaking (using all data including zeroes) and, on the other hand, copulation latency and copulation duration (only for the subset of replicates in which copulations occurred).

A separate additional analysis to investigate the inheritance of all behavioural traits compared the conspecific parental pairings (N, C) with the F_1 hybrid offspring in both directions (CxN, NxC) using one-way univariate ANOVA with analogous nesting, fixed and random factors as above, followed by post-hoc Tukey's tests. This qualitatively tested for deviations from the null expectation of intermediate inheritance, i.e. whether a trait shows dominance or maternal/paternal inheritance instead.

RESULTS

Mean values for all traits and pairings with 95% confidence intervals are reported in Table 3. Detailed ANOVA statistics and covariate effects (*F*-statistics, *p*-values, *β*-slopes) are reported in Appendix Table A2 to A7.

Baseline comparison of conspecific behaviour

Comparing the two species with four populations each as the baseline, *S. cynipsea* performed marginally more successful copulations per mating attempt than *S. neocynipsea* ($F_{1,6} = 6.44$, $P = 0.044$; Fig. 1a; appendix Table A3). Lower copulation frequencies were associated with more male mating attempts (covariate effect: $F_{1,150} = 12.18$, $P = 0.001$, $\beta = -0.365$; no other covariate had a significant effect). This variation in copulation frequency reflects corresponding species differences in mating interactions, as *S. neocynipsea* males performed more mating attempts per 30 minutes than *S. cynipsea* males ($F_{1,6} = 9.64$, $P = 0.021$; Fig. 1b), while *S. cynipsea* females displayed much more cumulative shaking (i.e. rejection or assessment behaviour: $F_{1,6} = 33.73$, $P = 0.001$; Fig. 1c). Both traits stimulate each other as the more male mating attempts necessarily entail more cumulative female shaking if the female is unwilling to mate (covariate effect: $F_{1,144} = 12.90$, $P < 0.001$, for females affecting males $\beta =$

0.136, for males affecting females $\beta = 0.606$). The first copulation started somewhat earlier in *S. neocynipsea* than in *S. cynipsea* ($F_{1,6} = 5.36$, $P = 0.055$; Fig. 2a), and copulation duration was slightly longer in *S. cynipsea* ($F_{1,6} = 4.48$, $P = 0.073$; Fig. 2b).

Baseline comparison of intercontinental S. neocynipsea behaviour

Copulation frequency (Fig. 1a) in cross-continental *S. neocynipsea* pairings did not vary among parentals, F_1 hybrids or backcrosses, nor did cumulative female shaking behaviour (Fig. 1c) and copulation latency (Fig. 2a). The only differences to be reported here are that males in cross-continental parental *S. neocynipsea* pairings performed more mating attempts than males in conspecific pairings within populations ($F_{1,6} = 12.09$, $P = 0.013$; Fig. 1b; appendix Table A4). Likewise, males of the cross-continental F_1 (hybrid) generation performed more mating attempts than males in the conspecific parental pairings ($F_{1,6} = 13.59$, $P = 0.009$; Fig. 1b). In both comparisons male mating attempts again covaried positively with female shaking (covariate effect: $F_{1,6} > 62.22$, $P < 0.001$, $\beta > 0.309$). Furthermore, copulation durations of F_1 hybrid pairings were longer than those of the parental conspecific pairings ($F_{1,6} = 15.39$, $P = 0.007$; Fig. 2b). Lastly, backcross direction showed no significant effect for any trait except copulation frequency ($F_{1,6} = 11.18$, $P = 0.012$), negatively affected by male mating attempts ($F_{1,53} = 25.491$, $P < 0.001$, $\beta = -0.556$) as well as female shaking duration ($F_{1,53} = 12.58$, $P = 0.001$, $\beta = -0.335$).

Heterospecific, F_1 hybrid, and backcross pairings

Heterospecific parental pairings never showed variation in crossing direction (CxN vs. NxC) in any trait, except for copulation latency ($F_{1,4} = 6.93$, $P = 0.013$; appendix Table A5). As expected, conspecific parental pairings resulted in much higher copulation frequencies than heterospecific pairings ($F_{3,12} = 18.01$, $P < 0.001$; Fig. 1a); copulation probability was additionally negatively related to the number of male mating attempts ($F_{1,362} = 18.00$, $P < 0.001$, $\beta = -0.186$; appendix Table A2). Even though there was only slight variation in the number of male mating attempts in an analogous test ($F_{3,12} = 3.32$, $P = 0.055$; Fig. 1b), females in conspecific pairings showed longer cumulative shaking duration than those in heterospecific pairings ($F_{3,12} = 13.05$, $P = 0.001$; Fig. 1c), the two traits again being correlated positively with each other ($F_{1,349} = 32.75$, $P < 0.001$, $\beta = 0.542$). Importantly, analogous multivariate

analysis of all these three traits together also indicated overall significant variation among species and cross types (C, N, CxN, NxC: $F_{9,36}$ = 13.19, P < 0.001). Copulation latency was much longer ($F_{3,10}$ = 9.46, P < 0.001), and copulation duration significantly shorter in heterospecific pairings ($F_{3,11}$ = 5.66, P = 0.006; Fig. 2a, b). Again, the corresponding multivariate analysis was also significant ($F_{6,21}$ = 6.29, P < 0.001).

Variation in F_1 hybrid direction (CxN vs. NxC; right grey vs. blue dots in Fig. 1c) was only evident for female shaking duration, with less shaking observed when the mother was *S. neocynipsea* ($F_{1,6}$ = 21.82, P < 0.001; appendix Table A6). This comparison confirms even the maternal inheritance of this trait (Fig. 3a), which is described in detail in the next section.

Backcrosses of F_1 hybrids with both parental species indicated no sex-specific variation for any of the studied behavioural traits, independent of whether the parental species was female or male (appendix Fig. A2; Table A7).

Inheritance of behavioural traits to the F_1 generation

Comparing the F_1 offspring, as depending on hybrid direction (i.e. CxN vs. NxC), with the parental species permits inferences about the inheritance of a trait (Fig. 3). One-way GLM with pure (baseline) parentals plus the reciprocal F_1 hybrids (CxN, NxC) as main effect with four levels revealed significant variation for female shaking duration suggesting partial maternal inheritance ($F_{3,12}$ = 23.33, P < 0.001; Fig. 3a); a post-hoc Tukey's test further revealed significant differences between parental species (appendix Table A1a). Analogous one-way GLM further showed significant variation for copulation frequency suggesting dominance of *S. neocynipsea's* low copulation pattern ($F_{3,12}$ = 3.77, P = 0.038; Fig. 3b), with the corresponding post-hoc Tukey's test shown in appendix Table A1b. All other traits showed no such variation suggesting the default intermediate inheritance (appendix Fig. A1).

Effects of biogeographic type

Biogeographic type (sym-, para-, allopatry) in the parental heterospecific pairings systematically affected copulation frequency, female shaking duration (Fig. 1a,c) and copulation latency (Fig. 2a), while the other traits showed no such variation. Copulation frequency and shaking duration increased from sympatric via parapatric to allopatric cross-continental pairings ($F_{4,6}$ = 11.78, P = 0.005 and $F_{4,6}$ = 4.97, P =

0.041, Fig. 1a; appendix Table A2). Similarly, flies of the cross-continental allopatric parental pairings required less time until first copulation ensued than flies from the corresponding European parapatric and sympatric pairings, although this appeared to be the case primarily in the CxN but not the NxC subset of the data ($F_{4,4} = 5.84$, $P = 0.001$; Fig. 2a). Crucially, the multivariate analysis subsuming all the traits presented in Fig. 1 also yielded a significant effect of biogeographic type ($F_{12,18} = 3.49$, $P = 0.008$), which was not quite the case for the two copulation traits presented in Fig. 2 ($F_{8,8} = 3.27$, $P = 0.057$).

Analogous analysis of the F1 hybrid generation only revealed systematic effects of biogeographic type on female shaking, however now showing a decrease in shaking duration from sympatric to allopatric pairings ($F_{4,6} = 4.48$, $P = 0.042$; Fig. 1c; appendix Table A3), but not on any other traits.

DISCUSSION

Sepsis cynipsea and *S. neocynipsea* were previously determined as different species based on their mitochondrial genetic distances (Su, Kutty, & Meier, 2008; Puniamoorthy, Su, & Meier, 2008), and behavioural as well as morphological differences (Pont & Meier, 2002). The species show low conspecific population differentiation but high heterospecific genetic differentiation based on neutral genetic microsatellite markers (Baur *et al.*, 2017, Chapter 1). Furthermore, North American and European populations of *S. neocynipsea* were recognized as the same species despite their geographical isolation and some morphological differences (Pont & Meier, 2002). We here documented quantitative differences in some precopulatory behavioural traits important for mating that are shared by both species, notably male mating attempts, female shaking behaviour, copulation frequency, latency, and duration. Particularly female shaking when males are mounted on their back, a trait that is part of the general repertoire of sepsid flies (Puniamoorthy *et al.*, 2009), is much more pronounced in *S. cynipsea* than in *S. neocynipsea*. Previous studies of *S. cynipsea* had identified this trait as contributing to female choice of mating partners and/or an expression of female reluctance to mate (Blanckenhorn *et al.*, 2000; Parker, 1972a,b; Ward, 1983; Ward, Hemi, & Rösli, 1992); it also has been shown to evolve in response to mating system manipulations due to sexual selection and conflict (Martin & Hosken, 2003).

We were here able to detect that females across almost all pairings and

generations respond with higher cumulative shaking to more male mating attempts, but this resulted in lower copulation frequencies, indicating reluctance to mate (Blanckenhorn et al., 2000). However, *S. cynipsea* females did shake longer in conspecific pairings than did *S. neocynipsea* females resulting in higher copulation frequencies suggesting mate assessment. Overall, if the female is willing to mate, males do not attempt to mount her so often, indicating females' willingness to copulate. Nevertheless, our evidence here that female shaking contributes to mate recognition, which could lead to reproductive isolation, is limited to significant variation in the expected direction between sym-, para-, and allopatric heterospecific pairings (Fig. 1c). It should be clear that traits merely differing quantitatively do not serve as well for reproductive isolation as do qualitatively different courtship traits (Puniamoorthy, 2014). Overall, our study revealed strong evidence for possible hybridisation of these two species, and also some evidence of species recognition and reproductive isolation at the precopulatory level, most apparent in terms of lower copulation frequencies and, particularly, in longer copulation latencies in heterospecific pairings. Under laboratory conditions, viable F_1 hybrid offspring had higher copulation success with each other and even with parental partners in backcrosses than occurred in the baseline conspecific parental pairings. This indicates hybrid vigour rather than outbreeding depression, facilitating hybridisation in nature (Todesco *et al.*, 2016; Wolf, Takebayasi, & Rieseberg, 2001). The extent to which hybridisation occurs in nature is currently being investigated at the genomic level.

Comparison of parental behaviour

Our study revealed anticipated but so far not quantified differences between the sister species in most assessed traits (Fig. 1), verifying on behavioural grounds that the two species are indeed separate but very closely related (Pont & Meier, 2002; Puniamoorthy, Su, & Meier, 2008; Su, Kutty, & Meier, 2008). Besides the prominent differences in female shaking behaviour discussed above, species differences in the other four traits assessed here are less pronounced. *Sepsis neocynipsea* males exhibited more mating attempts than *S. cynipsea* males. In nature, *S. cynipsea* is much more abundant than *S. neocynipsea* (and any other sepsid species) in places of co-occurrence in Europe, such as the Swiss Alps from where we sampled our populations (Pont & Meier, 2002). This implies that *S. cynipsea* males should more easily find conspecific mating partners, and therefore do not need to try hard to achieve

copulations. Despite more potential harassment of *S. neocynipsea* females by males of the other species in nature, known as the rare female hypothesis (Noor, 1995; Yukilevich, 2012), the latter performed less shaking but nevertheless ended up maintaining lower copulation frequencies, indicating that whatever other means *S. neocynipsea* females have to fend off unwanted males are very effective. *Sepsis cynipsea* females, in contrast, showed stronger shaking nevertheless resulting in more copulation per mating attempt, re-emphasizing the role of this behaviour in mate assessment (Ward, 1983).

Male and female traits depended significantly on each other, showing that the cumulative female shaking duration was longer the more often males attempted to mate with a partner, most likely to fend off the constant harassment of males. In turn, copulation frequency depended significantly on male-female interactions. Copulation success per mating attempt was lower in *S. neocynipsea*, for which ~35% of all male mating attempts resulted in copulations, as opposed to ~72% for *S. cynipsea* (Table 3). We recorded more copulations in conspecific pairings when males needed fewer attempts likely because females seem to be more willing to mate, possibly facilitated by species recognition of the conspecific partner. As expected, copulation success in our forced heterospecific pairings was much lower (~8%). Moreover, conspecific *S. cynipsea* pairings showed longer copulation latencies, suggesting *S. cynipsea* females spend overall more time assessing mates (by shaking more; Blanckenhorn et al., 2000), whereas *S. neocynipsea* females start copulating faster when mounted by a conspecific male. Finally, *S. cynipsea* showed slightly longer copulation durations (ca. 23 vs. 21 min), the biological significance of which is probably minor.

Perhaps surprisingly, *S. cynipsea* females shook less in heterospecific than conspecific pairings (Table 3). This lower shaking duration when facing heterospecific males could be a result of faster male dismounting or dislocation due to the species differences in the male armoured foreleg, which is an important male tool to cling on to the female's wing (Pont & Meier, 2002). In this context shaking appears effective for *S. cynipsea* females in assessing or rejecting mates (Blanckenhorn *et al.*, 1999, 2000; Martin & Hosken, 2003; Ward, 1983, Ward, Hemi, & Rösli, 1992), while *S. neocynipsea* females must have other means of assessing unwanted males: for instance surface or volatile hydrocarbons could be involved (Puniamoorthy, 2013). Other candidates could be subtle male courtship behaviour (e.g. circling around a female) or leg positions during pairing, which have been demonstrated in several

sepsid species and many other insects (Eberhard, 1996; Puniamoorthy *et al.*, 2009). Species recognition in heterospecific pairings here is most prominently expressed in longer copulation latencies compared to conspecific pairings. On the whole, the lower copulation frequencies, shorter copulation durations and longer copulation latencies in heterospecific pairings all signify strongly that these flies have more difficulties to mate, probably due to divergence in mate recognition systems.

Sepsis neocynipsea males in cross-continental, conspecific parental pairings performed more mating attempts, while not provoking more female shaking, perhaps because they dismounted faster on their own. On the other hand, similar female shaking durations, copulation frequencies, and latencies, with only minor differences in the other behavioural traits between the continents and across all generations, portend that *S. neocynipsea* from North America and Europe indeed recognize each other as the same species.

Trait inheritance in F_1 hybrids and hybrid vigour

Our study revealed no significant variation related to hybrid direction (CxN vs. NxC) for any behavioural trait, except for female shaking behaviour. Accordingly, F_1 hybrid offspring showed intermediate phenotypes relative to the parental species, indicating intermediate and presumably mainly autosomal inheritance of most of the quantitative behavioural traits considered here. Shaking behaviour is a prominent exception, which appears to be at least partly maternally inherited because hybrids expressed shaking more similar to the maternal species (Fig. 3a). Copulation frequency further showed evidence for dominance, as the lower copulation probabilities of *S. neocynipsea*, mediated by whatever mechanism, seem to be inherited by all the hybrids (Fig. 3b). Although our study design was not suited to calculate heritabilities, we were able to detect these strong signs of maternal inheritance and dominance. Further work to explore the genetic basis underlying these mechanisms can be a central aim of future studies. Our results confirm that most mating traits considered here are quantitative and heritable, and can therefore evolve in response to natural and sexual selection (cf. Martin & Hosken, 2003; Mühlhäuser & Blanckenhorn, 2004).

Interestingly, hybrid vigour was evident in F_1 hybrid offspring, not so much for the male and female behavioural traits themselves, but certainly by virtue of increased copulation success relative to the heterospecific parental pairings (Fig. 1, Table 3; Baranwal *et al.*, 2012). We can therefore conclude that hybridisation does

not immediately lead to cessation of mating behaviour and copulation in this system, although this may happen in later generations or further backcrosses. A first sign of mating barriers may be increased copulation durations of hybrids, suggesting some postmating but prezygotic difficulties such as disturbed sperm transfer (Arthur & Dyer, 2015). Whether reproductive success is depressed in hybrids or backcrosses despite the continuing mating success documented here will be investigated in future studies.

Comparing generations – are hybrids recognized?

Our data revealed little variation in most traits across the generations (parental, F_1, backcrosses), highlighting no breakdown over generations of important traits that potentially could reduce mating success. Instead, hybridisation may merely be disrupted by the difficulties in sperm transfer indicated by prolonged copulation durations of F_1 hybrids. Invariant male mating attempts also indicate that males do not discriminate strongly against heterospecific partners, as can be expected because sperm are relatively cheap, while effort in achieving a mating is substantial in any case (Birkhead & Moller, 1998). The mating system of both species is best described as scramble competition, with few if any aggressive interactions among males and a paramount role of female choice, by whatever mechanism (Blanckenhorn *et al.*, 2000).

Comparing the biogeographic range

Strongest precopulatory isolation is often demonstrated in sympatric species pairs, indicating reinforcement (Coyne & Orr, 1989, 2004; Yukilevich, 2012). A biogeographic effect in the parental pairings was detected for copulation frequency, latency, and female shaking behaviour (Figs. 1, 2). Heterospecific pairings from European populations in either sym- or parapatry exhibited longer latencies to copulation than flies in the cross-continental allopatric pairings, suggesting reinforcement of species recognition in areas where the two species co-occur. High conspecific gene flow may maintain this pattern throughout Europe (Fig. 1; Table 3). In contrast, heterospecific parental pairings showed stronger female shaking in allopatric pairings across continents and little shaking in sympatric pairings, though this pattern may be equally explained by faster dismounting of unwanted mates and species recognition of males in sympatric pairings. Reinforcement through stronger

female shaking behaviour in areas of sympatry is also reflected in the F_1 hybrid offspring, for which sympatric pairings showed more shaking than allopatric pairings (Fig. 1; Table 3).

CONCLUSIONS

We documented successful hybridisation under forced laboratory conditions between the close sister species *S. cynipsea* and *S. neocynipsea*. Female mate choice and species recognition can explain the low frequency of heterospecific relative to conspecific copulations realized per male mating attempt as well as their longer copulation latencies. The observed pattern of F_1 hybrids and backcrosses showing lower copulation frequencies, longer copulation latencies, and durations than the conspecific parentals, while at the same time achieving more copulations than flies in heterospecific pairings, could result from hybrid vigour mediated by mixture of genes from both species permitting species recognition (Baranwal *et al.*, 2012). We also observed heterospecific parental pairings with lower, and F_1 and backcross pairings with higher copulation durations as the parentals species, indicating possible difficulties with sperm transfer. Copulations do not necessarily imply successful fertilization, however, so offspring production needs to be documented to reveal possible mechanisms of post-copulatory isolation such as e.g. male sterility according to Haldane's rule (Haldane, 1922).

ACKNOWLEDGEMENTS

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Fig. 1. (a) Copulation frequency, (b) number of male mating attempts, and (c) cumulative female shaking duration (mean ± 95% CI population means) for parental conspecific, heterospecific, and F_1 hybrid offspring, representing all replicates (zeroes included). Conspecific parental pairings on the left are denoted in black for *S. cynipsea* and dark blue for *S. neocynipsea* populations. Heterospecific parental and F1 hybrid pairings for CxN are in grey, for NxC in light blue, while conspecific cross-continental *S. neocynipsea* (NxN) pairings are indicated in violet. Sympatric pairings are colored lighter than parapatric and allopatric, the latter being darkest.

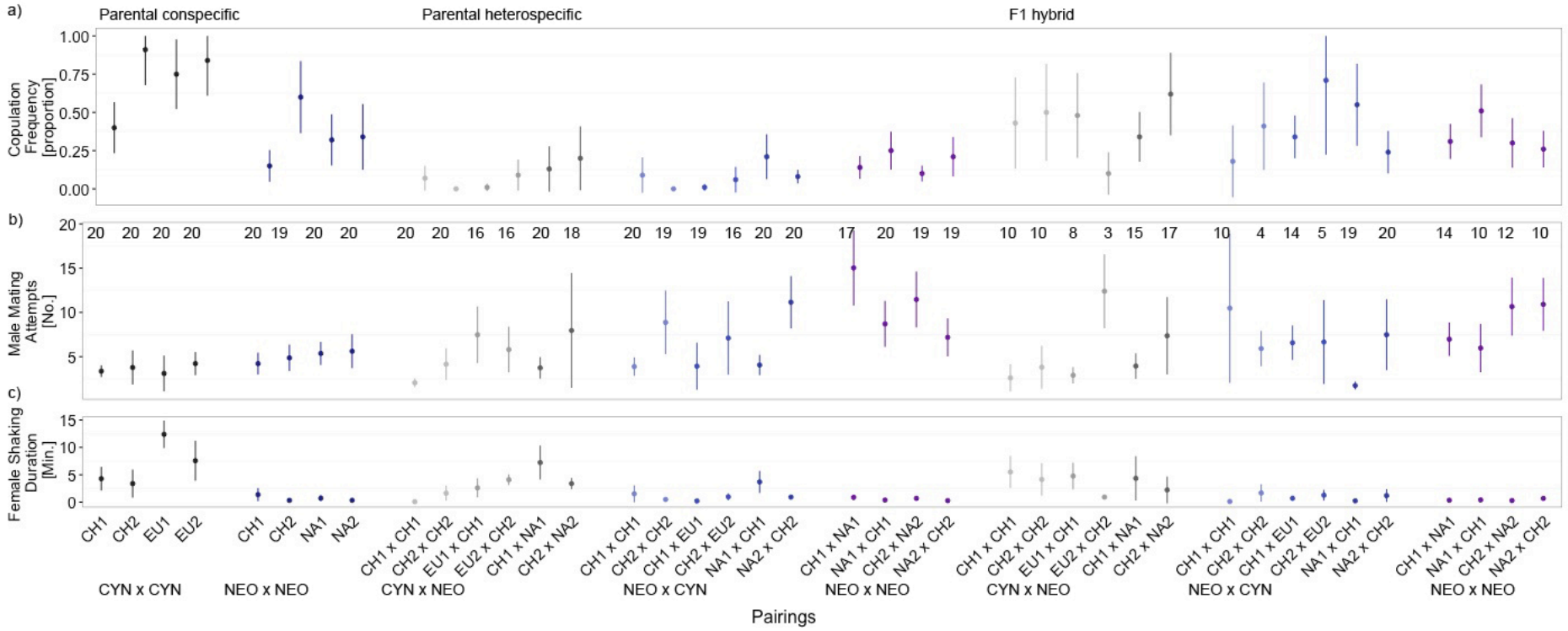

Figure 2. (a) Copulation latency and (b) copulation duration (mean ± 95% CI population means) for parental conspecific, heterospecific, and F_1 hybrid offspring, representing only replicates with at least one copulation. Conspecific parental pairings on the left are denoted in black for *S. cynipsea* and dark blue for *S. neocynipsea* populations. Heterospecific parental and F1 hybrid pairings for CxN are in grey, for NxC in light blue, while conspecific cross-continental *S. neocynipsea* pairings (NxN) are indicated in violet. Sympatric pairings are colored lighter than parapatric and allopatric, the latter being darkest.

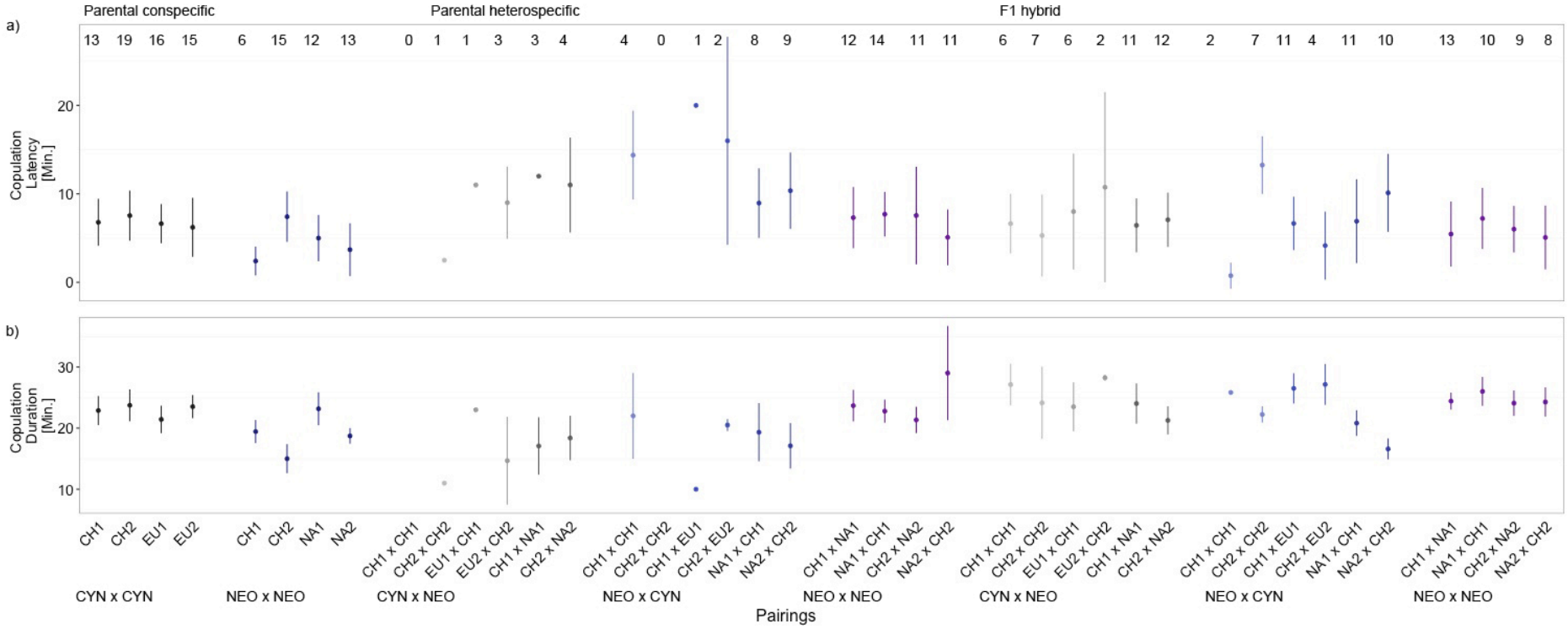

Figure 3. (a) Female shaking duration (indicating maternal inheritance) and (b) copulation frequency (indicating dominance) compared between conspecific *S. cynipsea* (black), *S. neocynipsea* (dark blue) and F_1 hybrid offspring (grey for CxN, light blue for NxC) pairings for evaluating the inheritance pattern of behavioural traits (mean ± 95% CI).

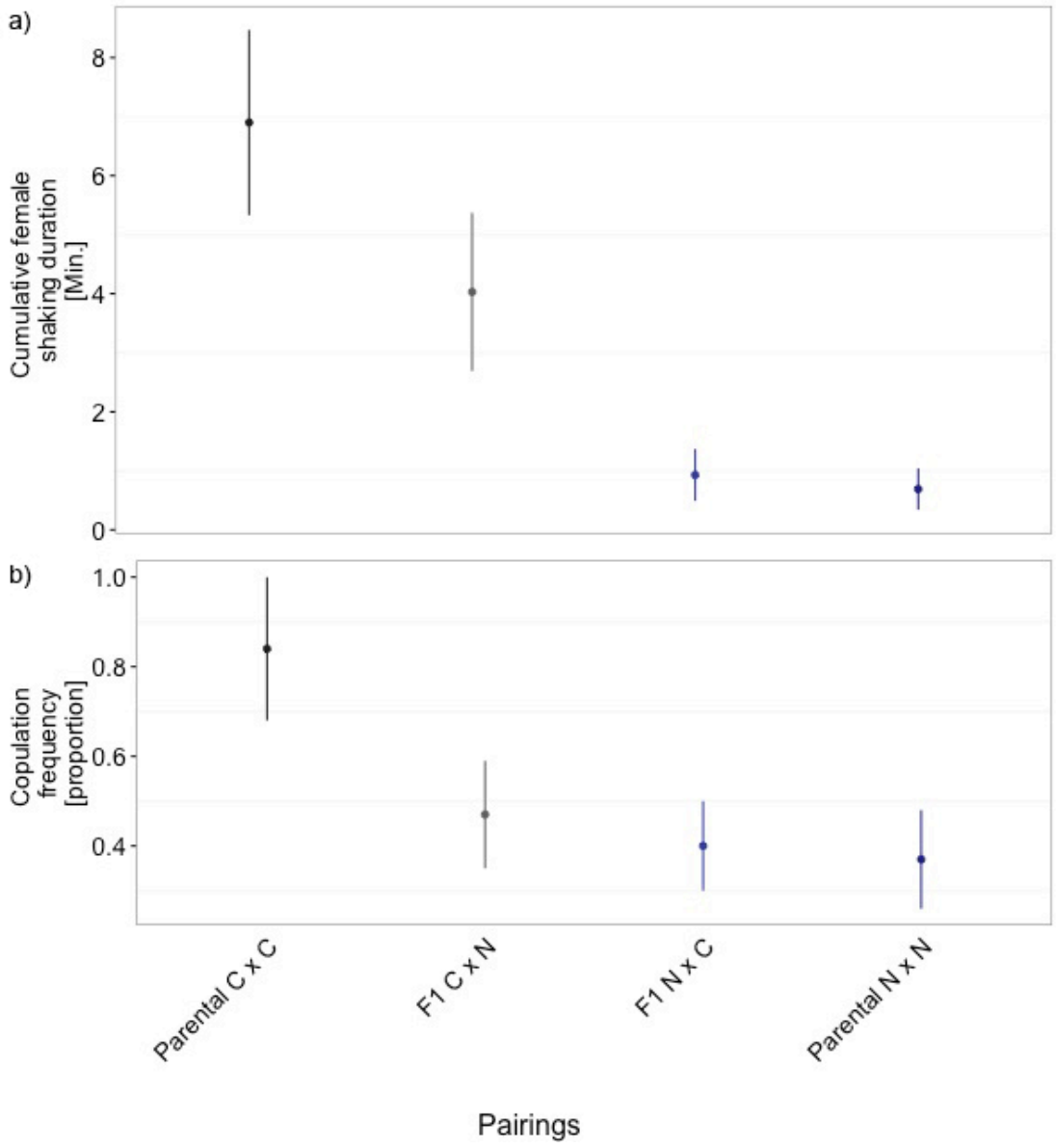

Table 1. Biogeographic origin of iso-female lines per population the study species.

Biogeographic origins (abbr.)		
S. cynipsea	*S. neocynipsea*	Coordinates
Switzerland, Zurich (CH_1)	Switzerland, Zurich (CH_1)	47°24'0.60"N, 8°34'23.97"E
Switzerland, Sörenberg (CH_2)	Switzerland, Sörenberg (CH_2)	46°49'23.72"N, 8°1'54.59"E
Scotland, Stirling (EU_1)		56°6'59.47"N, -3°56'12.83"W
Germany, Ludwigshafen (EU_2)		49°28'41.25"N, 8°22'21.65"E
	Idaho, Fort Hall (NA_1)	43°1'59.69"N, -112°26'17.91"W
	Wyoming, Lamar Valley (NA_2)	44°52'6.67"N, -110°10'28.72"W

EU = Europe; CH = Switzerland; NA = North America.

Table 2. Pairing scheme of three biogeographical types (female x male).

Biogeographical type	Pairings	Population replicates
Sympatry in Europe	EU *S. neocynipsea* x EU *S. cynipsea*	(CH1) x (CH1) (CH2) x (CH2)
Parapatry across Europe	EU *S. neocynipsea* x EU *S. cynipsea*	(EU1) x (CH1) (EU2) x (CH2)
Allopatry across continents:		
inter-specific	NA *S. neocynipsea* x EU *S. cynipsea*	(NA1) x (CH1) (NA2) x (CH2)
intra-specific	NA x EU *S. neocynipsea*	(NA1) x (CH1) (NA2) x (CH2)

Inter-specific (three groups and intra-specific (one group) pairing scheme. All pairings were reciprocal. Population abbreviations as in Table 1.

Table 3a. Mean values (± 95% CI) of all behavioural traits assessed.

Generation	Pairing	Male mating attempts [No.]	Female shaking duration [min]	Copulation frequency [proportion]	Copulation latency [min]	Copulation duration [min]
Parental	C*	3.61 ± 0.78	7.36 ± 1.59	0.72 ± 0.11	7.84 ± 1.38	22.92 ± 1.18
	C x N	5.04 ± 1.31	3.27 ± 0.88	0.09 ± 0.05	11.04 ± 2.63	16.92 ± 3.04
	N x C	6.48 ± 1.21	1.36 ± 0.52	0.08 ± 0.04	11.98 ± 2.61	18.59 ± 2.48
	N*	5.02 ± 0.76	0.70 ± 0.35	0.35 ± 0.10	6.11 ± 1.50	20.68 ± 1.01
	N_{EU}*	4.53 ± 0.96	0.86 ± 0.64	0.37 ± 0.15	6.99 ± 2.29	20.46 ± 1.78
	N x N	10.45 ± 4.21	0.55 ± 0.15	0.18 ± 0.05	7.97 ± 1.81	24.10 ± 2.11
	N_{NA}*	5.49 ± 1.15	0.54 ± 0.30	0.31 ± 0.13	4.84 ± 1.77	21.11 ± 1.68
F_1	C x N	5.04 ± 0.14	4.03 ± 1.34	0.45 ± 0.11	7.71 ± 1.72	23.33 ± 1.72
	N x C	5.68 ± 1.42	0.94 ± 0.45	0.36 ± 0.09	9.03 ± 1.95	22.28 ± 1.45
	N x N	8.57 ± 1.45	0.42 ± 0.12	0.34 ± 0.07	6.95 ± 1.70	24.71 ± 0.99

C = *S. cynipsea*; N = *S. neocynipsea*; EU = Europe; NA = North America; * denoting conspecific pairings; female x male.

Table 3b. Mean values (± 95% CI) of all behavioural traits assessed, regrouped by various criteria.

Generation	Pairing	Male mating attempts [No.]	Female shaking duration [min]	Copulation frequency [proportion]	Copulation latency [min]	Copulation duration [min]
Parental	Conspecific	4.31 ± 0.55	3.79 ± 0.94	0.54 ± 0.08	7.12 ± 1.03	21.98 ± 0.87
	Heterospecific	5.77 ± 0.89	2.30 ± 0.52	0.08 ± 0.03	10.87 ± 2.00	17.96 ± 1.92
	Sympatry	4.69 ± 1.14	1.00 ± 0.58	0.04 ± 0.04	13.00 ± 6.06	18.75 ± 5.97
	Parapatry	5.98 ± 1.59	1.89 ± 0.74	0.04 ± 0.03	13.86 ± 4.45	16.86 ± 4.35
	Allopatry	6.69 ± 1.83	3.91 ± 1.12	0.16 ± 0.07	11.21 ± 2.26	18.02 ± 2.20
F_1	Sympatry	5.11 ± 1.52	3.46 ± 1.46	0.42 ± 0.15	8.77 ± 2.58	24.51 ± 2.18
	Parapatry	6.19 ± 1.55	1.99 ± 0.97	0.40 ± 0.13	7.93 ± 2.59	25.99 ± 1.73
	Allopatry	5.17 ± 1.65	1.96 ± 1.24	0.44 ± 0.11	8.56 ± 1.90	20.79 ± 1.41
Backcrosses	F_1 x P	5.56 ± 0.94	0.87 ± 0.42	0.25 ± 0.08	7.02 ± 1.49	26.49 ± 1.43
	P x F_1	5.75 ± 0.85	1.26 ± 0.56	0.27 ± 0.09	8.41 ± 2.12	25.00 ± 1.94

female x male.

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APPENDIX

Figure A1. (a) Male mating attempts, (b) copulation duration, and (c) copulation latency compared between conspecific *S. cynipsea* (black), conspecific *S neocynipsea* (dark blue), and F_1 hybrid offspring (grey for CxN, light blue for NxC) pairings in evaluating the inheritance pattern of behavioural traits (mean ± 95% CI).

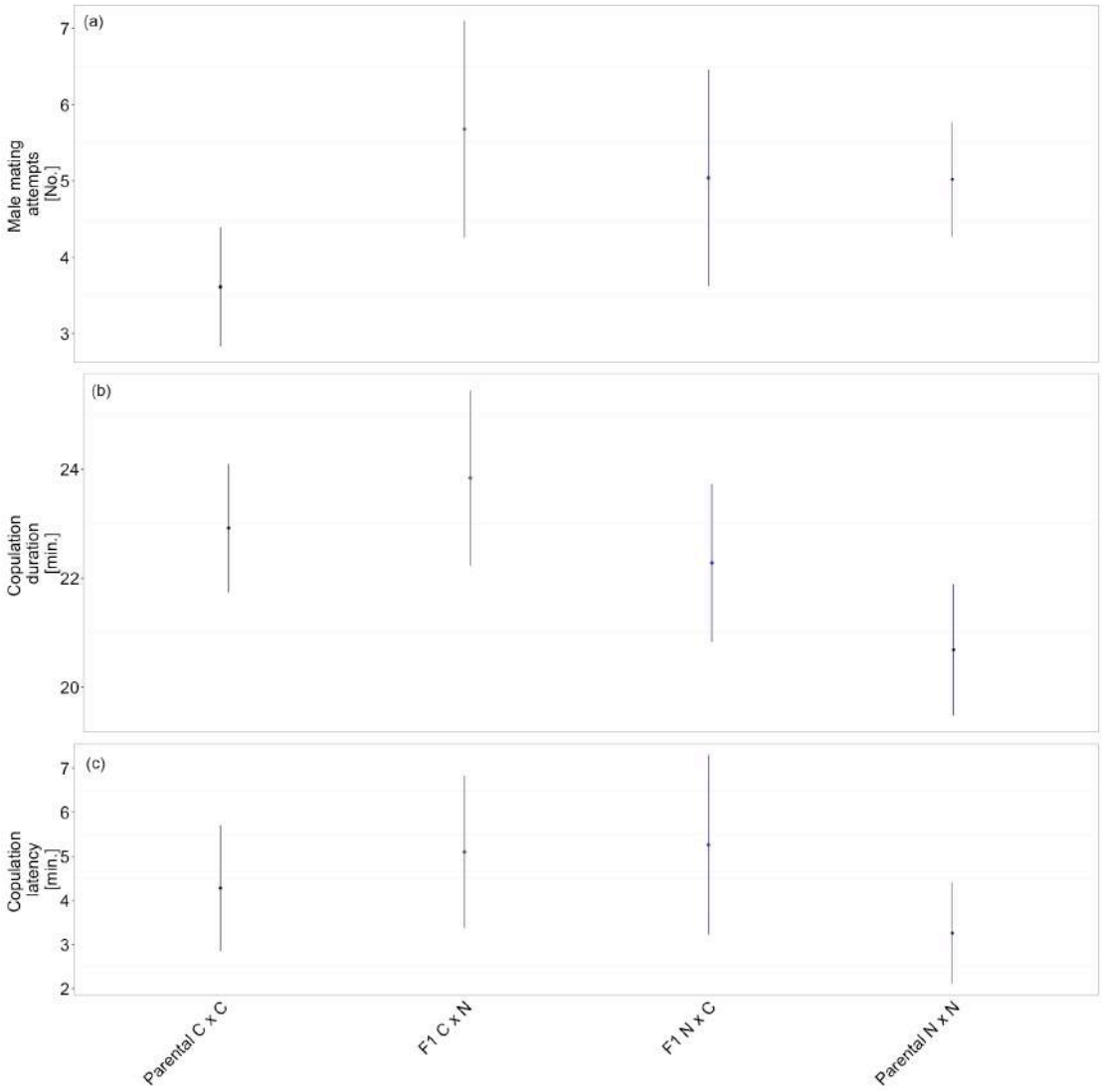

Figure A2. Comparing male and female behavioural traits of parental and backcross pairings (mean ± 95% CI). Backcrosses with CxN hybrids are denoted in green, those with NxC hybrids in blue. Continental *S. neocynipsea* (NxN) backcrosses are in pink.

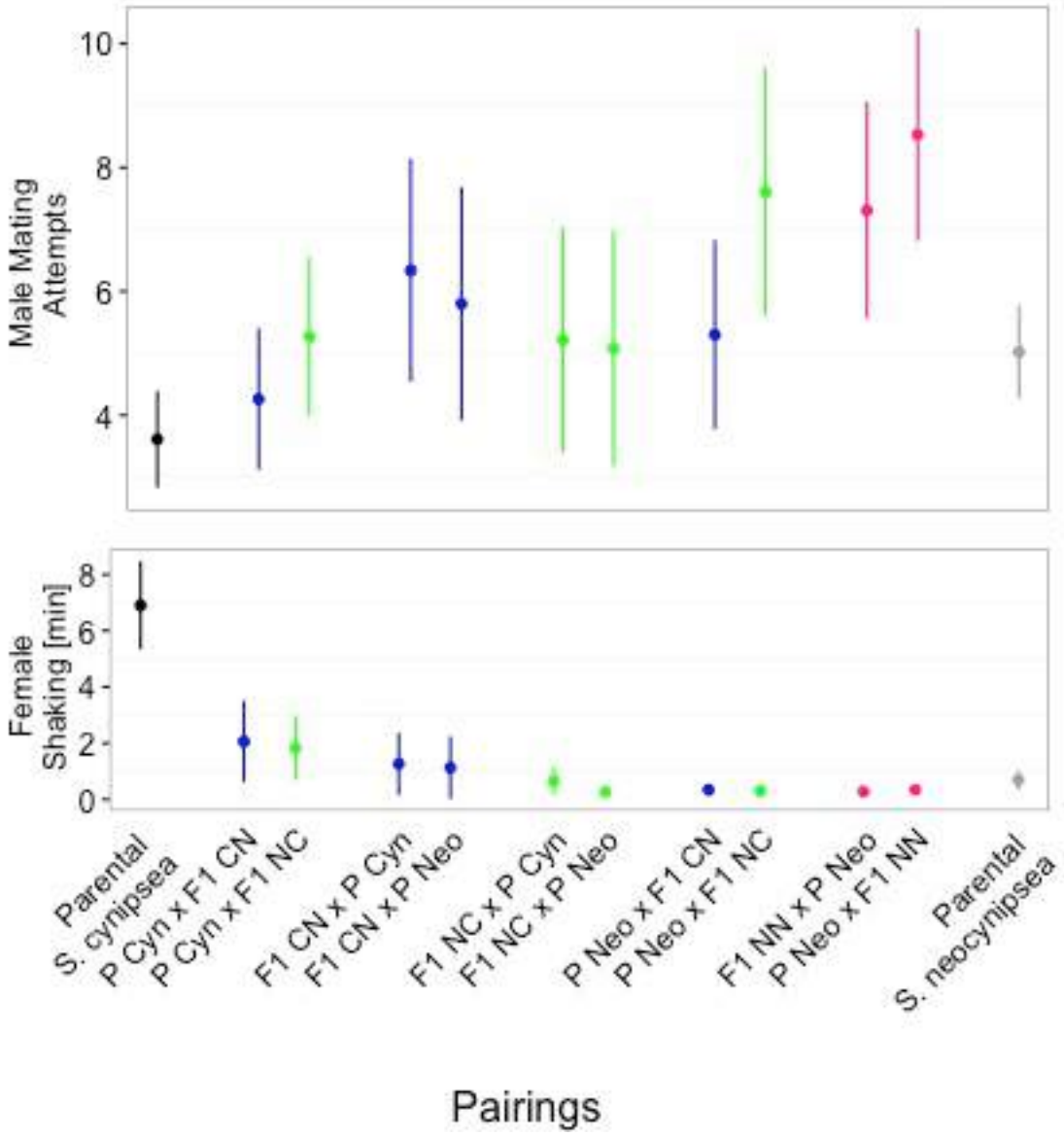

Figure A3. Comparing copulation traits of parental and backcross pairings (mean ± 95% CI). Backcrosses with CxN hybrids are denoted in green, those with NxC hybrids in blue. Continental *S. neocynipsea* (NxN) backcrosses are in pink.

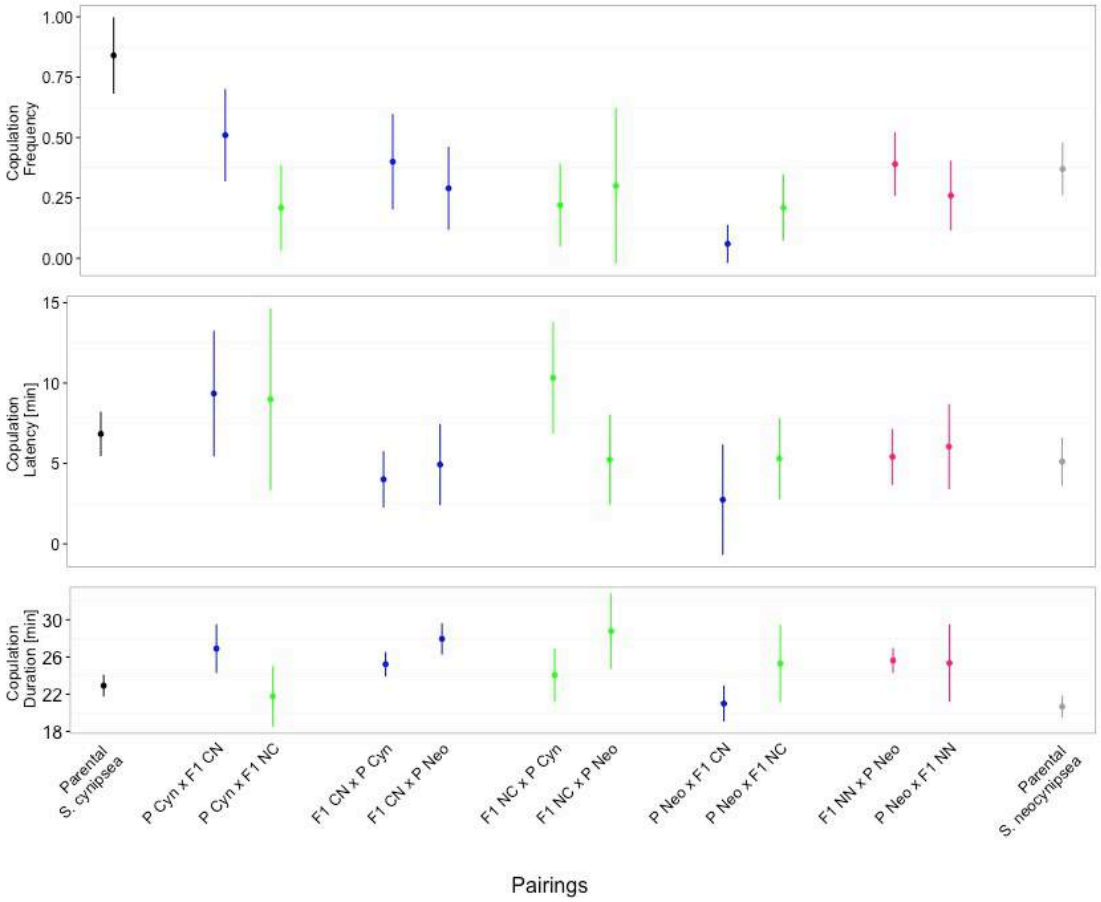

Table A1. Post-hoc paired Tukey's tests evaluating the inheritance pattern of behavioural traits.

Species	P C	F_1 C x N	F_1 N x C	P N
P C	-	0.262 ± 0.132	0.992 ± 0.128	1.205 ± 0.115
		p = 0.195	p < 0.001	p < 0.001
F_1 C x N	-0.270 ± 0.077	-	0.730 ± 0.142	0.943 ± 0.131
	p = 0.003		p < 0.001	p < 0.001
F_1 N x C	-0.326 ± 0.074	-0.056 ± 0.078	-	0.213 ± 0.127
	p < 0.001	p = 0.891		p = 0.986
P N	-0.373 ± 0.072	-0.103 ± 0.077	-0.470 ± 0.074	-
	p < 0.001	p = 0.537	p = 0.921	

C = *S. cynipsea*, N = *S. neocynipsea*, female x male: C x N, N x C. Post-hoc paired Tukey's tests following the one-way ANOVA comparing parental (P) conspecific (C, N) and F_1 hybrid pairings for evaluating the inheritance pattern of behavioural traits, calculated as the difference between the pairings ± standard errors. Female shaking duration [min] above; copulation frequency below the diagonal.

Table A2. ANOVA comparing con- and heterospecific parental pairings and nested biogeographic type.

Pairing	Comparison	Trait	*df*	*F*	*p*	*β*
Parental	C, N, CxN, NxC	No. male mating attempts	3, 12	3.32	***0.055***	
		/w female shaking duration	1, 349	32.752	**<0.001**	+0.158
		Female shaking duration	3, 12	13.047	**0.001**	
		/w male mating attempts	1, 349	32.752	**<0.001**	+0.542
		Copulation frequency	3, 12	21.488	**<0.001**	
		/w male mating attempts	1, 362	18.001	**<0.001**	-0.186
		Copulation latency	3, 10	9.462	**<0.001**	
		Copulation duration	3, 11	5.663	**0.006**	
	Biogeographic type (sym-, para-, allopatry)	No. male mating attempts	4, 6	0.619	0.665	
		Female shaking duration	4, 6	4.969	**0.041**	
		Copulation frequency	4, 6	11.782	**0.005**	
		Copulation latency	4, 4	5.844	**0.001**	
		Copulation duration	4, 5	0.811	0.522	

Bold values for $P < 0.05$; values in italics for $P < 0.1$. Univariate analyses of variance comparing con- and heterospecific parental pairings and nested biogeographic type for all behavioural traits (cross-continental *S. neocynipsea* pairings excluded). Only significant covariates are reported. Error degrees of freedom for the copulation traits are lower because some replicates featured no copulations whatsoever.

Table A3. ANOVA between parental *S. cynipsea* and *S. neocynipsea*.

Pairing	Comparison	Trait	*df*	*F*	*p*	*β*
Parental,	*S. cynipsea* vs.	No. male mating attempts	1, 6	9.641	**0.021**	
conspecific	*S. neocynipsea*	/w female shaking duration	1, 144	12.900	**<0.001**	0.136
		Female shaking duration	1, 6	33.727	**0.001**	
		/w male mating attempts	1, 144	12.900	**<0.001**	0.606
		Copulation frequency	1, 6	6.437	**0.044**	
		/w male mating attempts	1, 150	12.281	**0.001**	-0.365
		Copulation latency	1, 6	5.356	***0.055***	
		Copulation duration	1, 6	4.475	***0.073***	

Bold values for $P < 0.05$; values in italics for $P < 0.1$. Baseline planned sub-comparison of all behavioural traits between parental *S. cynipsea* and *S. neocynipsea* by univariate analyses of variance. Only significant covariates are reported.

Table A4. ANOVA between continental (EU vs. NA), and of cross-continental (NxN) vs. within-continental pairings of *S. neocynipsea*.

Pairing	Comparison	Trait	*df*	*F*	*p*	*β*
S. neocynipsea	parental	No. male mating attempts	1, 2	5.969	0.134	
	European vs.	Female shaking duration	1, 2	0.108	0.773	
	North American	Copulation frequency	1, 2	0.040	0.860	
		Copulation latency	1, 2	0.160	0.727	
		Copulation duration	1, 2	0.109	0.772	
	cross-continental	No. male mating attempts	1, 6	12.085	**0.013**	
	vs. continental	/w female shaking duration	1, 142	62.985	**<0.001**	+0.297
		Female shaking duration	1, 6	2.065	0.201	
		/w male mating attempts	1, 142	62.985	**<0.001**	+0.446
		Copulation frequency	1, 6	3.037	0.132	
		Copulation latency	1, 6	2.172	0.188	
		Copulation duration	1, 6	4.616	0.073	

Bold values for $P < 0.05$; values in italics for $P < 0.1$. Comparison of parental conspecific *S. neocynipsea* pairings across continents (EU vs. NA), and of cross-continental (NxN) vs. within-continental pairings by univariate analyses of variance for all behavioural traits.

Table A5. ANOVA between parental heterospecific pairings.

Pairing	Comparison	Trait	*df*	*F*	*p*	*β*
Parental, heterospecific	CxN vs. NxC	No. male mating attempts	1, 6	0.502	0.505	
		Female shaking duration	1, 6	4.533	***0.077***	
		Copulation frequency	1, 6	0.077	0.791	
		Copulation latency	1, 4	6.933	**0.013**	
		Copulation duration	1, 6	0.260	0.621	

Bold values for $P < 0.05$; values in italics for $P < 0.1$. Planned sub-comparison of all behavioural traits according to the direction of heterospecific parental pairing (CxN vs. NxC) by nested univariate analyses of variance. No significant covariates.

Table A6. ANOVA between heterospecific F1 hybrid directions and biogeographic type.

Pairing	Comparison	Trait	*df*	*F*	*p*	*β*
F1	CxN vs. NxC	No. male mating attempts	1, 6	1.178	0.311	
		/w female shaking duration	1, 88	6.405	**0.013**	+0.134
		Female shaking duration	1, 6	21.821	**0.001**	
		/w male mating attempts	1, 88	6.405	**0.013**	+0.507
		Copulation frequency	1, 6	0.452	0.512	
		Copulation latency	1, 6	0.214	0.657	
		Copulation duration	1, 6	1.099	0.325	
	Biogeographic type (sym-, para-, allopatry)	No. male mating attempts	4, 6	1.370	0.342	
		Female shaking duration	4, 6	3.349	***0.075***	
		Copulation frequency	4, 6	3.137	***0.072***	
		Copulation latency	4, 6	0.271	0.887	
		Copulation duration	4, 6	4.480	**0.042**	

Bold values for $P < 0.05$; values in italics for $P < 0.1$. Univariate analyses of variance comparing hybrid F1 pairings and nested biogeographic type for all behavioural traits (cross-continental *S. neocynipsea* pairings excluded). Only significant covariates are reported.

Table A7. ANOVA between all heterospecific backcross directions.

Pairing	Comparison	Trait	*df*	*F*	*p*	*β*
Backcrosses	All backcross types	No. male mating attempts	7, 16	1.728	0.163	
		Female shaking duration	7, 12	1.574	0.216	
		Copulation frequency	7, 16	1.003	0.459	
		Copulation latency	7, 10	1.600	0.223	
		Copulation duration	7, 10	1.521	0.243	
	PxF_1 vs. F_1xP	No. male mating attempts	1, 6	1.008	0.328	
		Female shaking duration	1, 6	2.616	0.139	
		Copulation frequency	1, 6	0.063	0.805	
		Copulation latency	1, 6	1.256	0.287	
		Copulation duration	1, 6	0.931	0.357	

Bold values for $P < 0.05$; values in italics for $P < 0.1$. Comparison of all behavioural traits for all backcross types and backcross direction (PxF_1 vs. F_1xP) by univariate analyses of variance.

CHAPTER THREE

Patterns of postzygotic isolation between two closely related dung fly species (*Sepsis cynipsea* and *S. neocynipsea*; Diptera: Sepsidae)

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ABSTRACT

Identifying the contribution of pre- and postzygotic barriers to gene flow is a key purpose of speciation research. The closely related dung fly species *Sepsis cynipsea* and *S. neocynipsea* offer great opportunity to address these issues as these lineages show incomplete pre-mating isolation and signatures of reinforcement in areas of sympatry. Here, we examined the role of postcopulatory isolating barriers by comparing female fecundity and egg-to-adult viability of F_1 and F_2 hybrids, as well as backcrosses of F_1 hybrids with the parental species, via replicated crosses of sym-, para-, and allopatric populations. Egg-to-adult viability was significantly but not totally suppressed in hybrids, and offspring production approached nil in the F_2 generation (hybrid breakdown). This indicates intrinsic incompatibilities related to yet unspecified barriers in sperm transfer, difficulties to form a zygote, and/or a decreased survival of juvenile stages. Moreover, viable F_1 hybrid offspring showed almost absolute male (the heterogametic sex) sterility while females remained largely fertile, in accordance with Haldane's rule. Crossing European and North American *S. neocynipsea* indicated similar but much weaker isolating barriers developing between them, which are most easily explained by random processes (i.e. genetic drift). Hybridisation between the two species in European areas of sympatry (here the Swiss Alps) is prevented to some degree by reinforcement, thus implicating natural selection.

INTRODUCTION

Speciation of genetically diverged populations is driven by ecological, spatial, or temporal niche differentiation that ultimately leads to reproductive incompatibilities through selection or genetic drift (Coyne & Orr, 1997, 2004; Dobzhansky, 1951; Mayr, 1942; Schluter 2000, 2001). Heterospecific mating may then be inhibited through behavioural, morphological, or olfactory differences leading to strong prezygotic barriers, as predicted in the biological species concept (Futuyama, 1986; Mayr, 1940, 1982). However, closely related species may show incomplete prezygotic reproductive isolation, forming hybrid progeny with a reduced fitness, hybrid sterility, or low viability compared to their parental species as a result of postzygotic isolation (Hood, Egan, & Feder, 2012; Reed & Markow, 2004; Wassermann & Koepfer, 1977). Due to genetic interactions between sex chromosomes, partially recessive alleles of hybrid progeny can particularly reduce

fitness in the heterogametic sex according to Haldane's rule (Coyne, 1985; Haldane, 1922; Turelli & Orr, 1995). On the other hand, F_1 hybrids can show hybrid vigour due to masking of deleterious, recessive alleles, which subsequently may (or not) result in reduced fitness of F_2 progeny. This implicates epistasis in the process of hybrid breakdown, resulting in formation of viable F_1 hybrid offspring but no F_2 hybrids (Burton, Ellison, & Harrison, 2006; Dobzhansky, 1936; Endler, 1977; Muller 1942).

The closely related sister species *Sepsis cynipsea* and *S. neocynipsea* (Diptera: Sepsidae) offer great opportunity to investigate evolutionary mechanisms underlying reproductive barriers in recently diverged species. Precopulatory barriers between the species are evident in various behavioural traits such as male mating attempts, female shaking to fend off males, or copulation frequency, which is partly driven by character displacement and reinforcement in areas of sympatry (Giesen, Blanckenhorn, & Schäfer, 2017, Chapter 2). That is, our behavioural studies indicate that these two species hybridise at least in the laboratory. However, this prezygotic isolation is not absolute, though very strong, so that postcopulatory barriers to gene flow should play an additional role separating the gene pools of the two species.

We thus here investigated female fecundity and egg-to-adult viability in con- vs. heterospecific parental crosses, F_1, and F_2 hybrids, as well as backcrosses of F_1 hybrid offspring with their parental species. We assessed a number of typical traits for estimating postmating isolating barriers: female age at first reproduction, the probability of producing eggs (p(eggs)), and egg number of the first clutch as indicators of fecundity, as well as the probability of producing offspring (p(offspring)) and offspring number of the first clutch. The number of offspring per eggs laid allowed additional calculation of egg-to-adult viability. Since crosses were performed bidirectionally, we also obtained information on male vs. female fertility.
We hypothesized that female fecundity and egg-to-adult viability would be significantly lower in heterospecific parental, hybrid, and backcrosses compared to their conspecific parental crosses, due to intrinsic incompatibilities such as difficulties with sperm transfer, unsuccessful fertilization, and/or decreased hybrid viability. Moreover, we expected lower fecundity and viability in backcrosses with a hybrid male and a parental female relative to the converse, in accordance with Haldane's rule, as the heterogametic sex of hybrids is often sterile (Haldane, 1922) because sex chromosomes tend to be frequently involved in reproductive isolation, resulting in genetic incompatibilities between the species (Coyne & Orr, 1989). As our previous

behavioural study demonstrated fewer prezygotic barriers in allopatric and parapatric relative to sympatric crosses (Giesen, Blanckenhorn, & Schäfer, 2017, Chapter 2), and because genetic population differentiation between intercontinental *S. neocynipsea* shows divergence similar to that between the two species in Europe (Baur *et al.*, 2017), it is also of great interest to analyse fecundity and viability of intercontinental *S. neocynipsea* crosses in further exploring ongoing speciation processes.

MATERIALS & METHODS

Study organism

The two closely related species *S. cynipsea* and *S. neocynipsea* (Diptera: Sepsidae) exhibit only little differentiation between species on the mitochondrial barcoding genes COI and CytB (Su, Kutty, & Meier, 2008). However, population genetic differentiation revealed fairly high heterospecific (European pairwise F_{ST}=0.16) as well as cross-continental differentiation (F_{ST}=0.19 between North American *S. neocynipsea* and European *S. cynipsea*, and F_{ST}=0.10 between continental *S. neocynipsea* populations; Baur *et al.*, 2017, Chapter 1), while gene flow within the European species is high (mean F_{ST} ~0.03). Moreover, the species show strong similarities in morphology, behaviour, and ecology (Pont & Meier, 2002). *Sepsis cynipsea* is the most abundant sepsid in north-central Europe, where it occurs in sympatry with the rare *S. neocynipsea* in some mountainous regions such as the Swiss Alps. In contrast, the latter species occupies the same warm-adapted temperature niche in North America, where *S. cynipsea* does not occur (Pont & Meier, 2002). The mating system of *S. cynipsea* has been described in detail (Blanckenhorn *et al.*, 1999, 2000; Parker, 1972a, b; Puniamoorthy *et al.*, 2009; Ward, 1983; Ward, Hemmi, & Rösli, 1992), while relatively little is known about *S. neocynipsea* (Eberhard, 1999; Puniamoorthy *et al.*, 2009; Rohner, Blanckenhorn, & Puniamoorthy, 2016). A detailed description of the maintenance of the flies in our laboratory and an ethical note can be extracted from Chapter 2.

Crossing scheme

We caught gravid females from six sites (i.e. populations) to establish 5 to 15 iso-female lines per population in the laboratory. Sympatric populations were collected in Switzerland (Zurich: 47°24'0.60"N, 8°34'23.97"E; Sörenberg: 46°49'23.72"N,

8°1'54.59"E), where both species co-occur. We further obtained *S. cynipsea* from two parapatric European regions (Ludwigshafen, Germany: 49°28'41.25"N, 8°22'21.65"E; Stirling, Scotland: 56°6'59.47"N, -3°56'12.83"W), where *S. neocynipsea* only has been observed in adjacent populations (Ozerov, 2005; Pont & Meier, 2002). The other *Sepsis neocynipsea* were collected from two allopatric North American populations (Fort Hall, Idaho: 43°1'59.69"N, -112°26'17.91"W; Lamar Valley, Wyoming: 44°52'6.67"N, -110°10'28.72"W), where *S. cynipsea* does not exist.

Flies from iso-female lines, kept for variable periods of time in the laboratory, were used in conspecific parental crosses within each of the four populations per species as the baseline. They were further used in two replicate reciprocal population crosses of European and North American *S. neocynipsea* (conspecific allopatric cross-continental crosses). Moreover, reciprocal heterospecific parental crosses of three biogeographic types were formed with two population replicates each: European sympatry, European parapatry, and cross-continental allopatry (Table 1). One population replicate consisted of 15 to 20 replicate crossings of iso-female lines.

Hybrid F_1 and F_2 flies for our assessments were generated by randomly combining up to 20 flies of each sex from various iso-female lines of a given population and species to be paired with so selected flies from the other species, all done reciprocally. Matings in these settings were thus necessarily heterospecific. Potentially lower sample sizes with F_1 and F_2 hybrid offspring were expected due to difficulties in obtaining hybrids. For backcrosses we targeted a sample size of six replicates per pairing, as we set up two reciprocal types (female hybrid with male parental – F_1xP, and female parental with male hybrid – P x F_1) to detect possible sex specific effects.

We obtained fecundity and egg-to-adult viability measurements for (1) con- and heterospecific parental crossings (P, N≈18 per crossing replicate), (2) F_1 hybrid crossings using the resulting offspring of heterospecific crossings (N≈10 per crossing replicate), (3) F_2 hybrid crossings (N≈6, per crossing replicate), and (4) backcrosses of F_1 hybrid offspring with the parental species (BC, N≈6, per crossing replicate). All crosses were done reciprocally.

Fecundity and egg-to-adult viability assessment

One cross consisted of one female and two males (either con- or heterospecific). This grouping should simulate natural conditions, increasing hybridization probability according to Puniamoorthy (2014), and limiting the probability of total failure due to male sterility. Virgin flies were assigned randomly after adult eclosion to a round 50 mL glass vial containing fresh cow dung as oviposition substrate in a plastic rectangular dish (4.2 cm x 2.1 cm x 1.6 cm) plus some grains of sugar. We scored (i) female age when she first laird eggs into the dung [in days], (ii) her first clutch size, (iii) the number of emerged offspring, plus (iv) female and (v) male mating partner head widths as measures of their body size (Blanckenhorn, Reusch, & Mühlhäuser, 1998; Rohner, Blanckenhorn, & Puniamoorthy, 2016). From these observations we further derived (vi) the proportion of females (of a given cross) that produced eggs (p(eggs)) [Y/N] and (vii) offspring (p(offspring)) [Y/N]. Lastly we calculated (viii) egg-to-adult viability as the proportion of offspring emerging from a clutch. Note that given our set-up with lots of heterospecific crosses, we expected low overall numbers of fertile eggs and offspring numbers.

Statistical analyses

All traits were analysed separately with female head width as covariate because body size typically affects fecundity measures (Honek, 1993; only significant covariates are reported in the Results) in SPSS Statistics Version 23, using univariate GLM with binomial errors when outcome variables were binary or proportional. A given trait was analysed as a function of species (C: *S. cynipsea*, N: *S. neocynipsea*, CN and NC; or, analogously, N_{EU}, N_{USA}, $N_{EU}N_{USA}$ and $N_{USA}N_{EU}$ – females always first), biogeographic type nested in species (sympatric vs. parapatric vs. allopatric), all as fixed factors, and population nested within biogeographic type and species as a random effect. Additional planned baseline comparisons were performed to compare the two parental species (C vs. N), the continental *S. neocynipsea* crosses (N_{EU} vs. N_{USA}), and the direction of heterospecific mating (CN vs. NC; $N_{EU}N_{USA}$ vs. $N_{USA}N_{EU}$). F_1, F_2 hybrid and backcrosses were analysed analogously. Lastly, we compared the hybrid offspring (F_1 or F_2 hybrid) with their parental, conspecific crosses. For some comparisons no statistical analyses were applicable due to a lack of data (i.e. no emerged hybrid offspring).

RESULTS

Table 2 reports mean values for egg-to-adult viability, no. and probability of eggs and offspring produced, age at first reproduction, and male and female head width with 95% confidence intervals and corresponding GLM (χ^2-statistics, p-values) for all comparisons.

Baseline comparison between conspecific S. cynipsea and S. neocynipsea

Most fecundity traits did not differ between the two species (Table 2, Fig. 1). Egg-to-adult viability of offspring was around 30% ($\chi^2(1,6) = 1.41$, $P = 0.235$, Fig. 1e). Females laid their first eggs after ca. six days ($\chi^2(1,6) = 2.04$, $P = 0.154$, Fig. 2a) with a mean clutch size of ~45 eggs ($\chi^2(1,6) = 1.34$, $P = 0.247$, Fig. 1b). In total, ca. 68% of all females laid eggs (p(eggs): $\chi^2(1,6) = 0.04$, $P = 0.846$, Fig. 1a). Offspring number was around ~22 individuals ($\chi^2(1,6) = 0.37$, $P = 0.542$, Fig. 1d). The only difference between the species was evident in the probability of producing offspring, which was higher for *S. cynipsea* (p(offspring): 0.700 ± 0.128) than for *S. neocynipsea* (0.545 ± 0.149, $\chi^2(1,6) = 3.87$, $P = 0.049$, Fig. 1c), despite the latter species being bigger (females: 0.79 ± 0.01 mm, males: 0.76 ± 0.01 mm) than the former (females: 0.74 ± 0.01, $\chi^2(1,6) = 66.67$, $P < 0.001$, Fig. 2b; males: 0.71 ± 0.01, $\chi^2(1,5) = 7.77$, $P = 0.005$, Fig. 2c).

Baseline comparison between continental S. neocynipsea

Differences between European and North American *S. neocynipsea* were only found in the female's age of first reproduction ($\chi^2(1,2) = 26.51$, $P < 0.001$) with North American *S. neocynipsea* females (6.55 ± 0.69) being older when first laying a clutch than those from Europe (4.91 ± 0.18), although females were of the same size (0.79 ± 0.01, $\chi^2(1,2) = 0.00$, $P = 0.999$). All other traits were similar: both laid around ~45 eggs per clutch ($\chi^2(1,2) = 0.82$, $P = 0.365$, Fig. 3b) with p(eggs) around ~68% ($\chi^2(1,2) = 0.21$, $P = 0.646$, Fig. 3a). From these clutches ca. 23 offspring emerged ($\chi^2(1,2) = 0.15$, $P = 0.702$, Fig. 3d), p(offspring) being ca. ~55% ($\chi^2(1,2) = 0.60$, $P = 0.440$, Fig. 3c). The egg-to-adult viability found on both continents was roughly ~23% ($\chi^2(1,2) = 1.10$, $P = 0.295$, Fig. 3e).

Intercontinental S. neocynipsea crosses over three generations

Although no differences in crossing direction across continents were detectable for *S. neocynipsea*, p(eggs) was higher when females from North America and males from Europe were crossed (~59%) relative to the reciprocal cross (~35%, $\chi^2(1,2) = 3.13$, $P = 0.007$, Fig. 3a). In the intercrossed F_1 generation only the female age at first reproduction differed significant in the crossing direction showing a difference of one day (χ^2 (1,2) = 8.33, $P = 0.004$). Cross-continental F_2 hybrids showed an asymmetry in hybridization direction with crosses of European females and North American males resulting in no eggs and no offspring whatsoever, although the overall sample size was very low (N=3, Table 2, Fig. 3e). Backcrosses showed no significant differences across all measured traits (Table 2, Fig. 3).

Fitness of heterospecific crosses and their hybrid offspring

In parental heterospecific crosses, the probability of producing eggs was affected by hybridisation direction (CN vs. NC) with CN crosses (0.41 ± 0.09) having lower p(eggs) than NC (0.64 ± 0.09, $\chi^2(1,6) = 8.05$, $P = 0.005$; Fig. 1a). In this specific case, this trait was also affected by biogeographic type ($\chi^2(4,6) = 32.96$, $P < 0.001$), with sympatric crosses resulting in lowest p(eggs) ~ 27%, while parapatric (~65%) and allopatric (~70%) resulted in much higher egg production.

Pairwise comparison of con- and heterospecific parental crosses revealed expectedly higher egg-to-adult viability in conspecific (~30%) than in heterospecific (~4%) crosses ($\chi^2(1,14) = 50.70$, $P < 0.001$, Fig. 1e). However, the same pairwise comparison showed no difference for p(eggs) between con- (~68%) and heterospecific (~53%) crosses ($\chi^2(1,15) = 0$, $P = 0.99$; Table 2; Fig. 1a), while the probability of producing offspring differed between the conspecific (~63%) and heterospecific (~8%) crosses (no statistics applicable; Fig. 1c).

Crossing of F_1 hybrid offspring produced F_2 hybrid offspring only in the NC direction (~13 offspring), with CN crosses generating no offspring whatsoever ($\chi^2(1,3) = 5.10$, $P < 0.001$, Fig. 1d). Consequently, the probability of producing offspring (no statistics applicable; Fig. 1c) and egg-to-adult viability (no statistics applicable; Fig. 1e) were similarly affected.

F_1 hybrid females and males resulting from heterospecific CN crosses were larger than those from heterospecific NC crosses, a significant effect of crossing

direction (females: $\chi^2(1,4) = 4.91$, $P = 0.027$, Fig. 2b; males: $\chi^2(1,4) = 6.56$, $P = 0.010$, Fig. 2c). As the egg-to-adult viability from all F_1 hybrid crosses was significantly reduced in the CN direction, it was much lower than that of the conspecific parental crosses ($\chi^2(1,10) = 30.64$, $P < 0.001$, Fig. 1e). Interestingly, F_2 hybrids of both sexes were significantly smaller than the parental females (females: $\chi^2(1,6) = 60.11$, $P < 0.001$, Fig. 2b; males: $\chi^2(1,6) = 33.87$, $P < 0.001$, Fig. 2c).

We found the same hybridisation asymmetry in the next F_2 hybrid generation. Adults emerged only from one clutch out of 10 replicates for the CN direction, while ~38% p(offspring) resulted from NC crossings (no statistics applicable; Fig. 1c). This similarly affected offspring number ($\chi^2(1,2) = 7.58$, $P = 0.006$, Fig. 1d) and egg-to-adult viability (no statistics applicable; Fig. 1e). Compared to the conspecific crosses (~30%), the F_2 hybrid viability was significantly decreased to ~10% ($\chi^2(1,6) = 4.99$, $P = 0.025$, Fig. 1e).

Detecting Haldane's rule in backcrosses of F_1 hybrids with the parental species

To test sex-specific deleterious hybridization effects, we reciprocally crossed F_1 hybrids with parental species. Egg-to-adult viability was decreased when hybrid males mated with parental females ($\chi^2(1,12) = 67.37$, $P < 0.001$, Fig. 1e), but not vice versa. Egg ($\chi^2(1,12) = 16.57$, $P < 0.001$, Fig. 1b) and offspring numbers including zeroes ($\chi^2(1,12) = 54.30$, $P < 0.001$) were similarly decreased for this group.

DISCUSSION

The traditional biological species concept only allows for interbreeding between populations within species, while gene flow across species should be inhibited via isolating mechanisms (Futuyama, 1986; Mayr, 1940, 1982). This limitation of gene flow within species boundaries is similar across many speciation models, such as the evolutionary species concept, which follows the assumption that a lineage that evolved from a most common recent ancestral lineage is separate from others, and consequently exhibits its own evolutionary role (Simpson, 1961). In most species concepts gene exchange between species or lineages is more or less excluded, but after more extensive research on the speciation process scientists discovered that hybridisation between species is not uncommon (Anderson, 1949; Barton & Bengtsson, 1986; DeMarais *et al.*, 1992; Mallet, 2007; Nolte & Tautz, 2010;

Rieseberg *et al.*, 2003; Trier *et al.*, 2014). With this new research, the concept of hybridization was also seen to play a role in the isolation of species, as it is assumed that gene exchange between species always is at least limited, if not completely blocked, by reproductive isolating mechanisms (Dobzhansky, 1970). Therefore, reproductive isolating mechanisms play a central role in the speciation process, as hybridization between incipient species is to some extent inhibited via genetic incompatibilities reflected in Haldane's rule, F_2 hybrid breakdown, or hybrid asymmetry, which are all discussed below.

Previous studies have shown that *Sepsis cynipsea* and *S. neocynipsea* differ in their behavioural and morphological traits (Pont & Meier, 2002; Giesen, Blanckenhorn, & Schäfer, 2017, Chapter 2), with some genetic differentiation also evident based on the COI and CytB gene sequences (Su, Kutty, & Meier, 2008) and population genetic data (Baur *et al.*, 2017, Chapter 1). Despite genetic differences of similar magnitude between North American and European *S. neocynipsea* populations (Baur *et al.*, 2017, Chapter 1), these continental populations are recognized as the same species based on great similarities in morphology and behaviour (Pont & Meier, 2002; Giesen, Blanckenhorn, & Schäfer, 2017, Chapter 2). Forced interbreeding under laboratory conditions between the two species here resulted in successful hybridization, however with often strongly reduced hybrid fitness due to hybrid breakdown. We quantified the degree of postcopulatory isolation by measuring fecundity, fertility and egg-to-adult survival, leading us to identify genetic incompatibilities between the two species. We detected disruption in one of the heterospecific hybridization directions (CN) in the probability of producing eggs, as well as a significant breakdown of viability in the later hybrid generations. Male F_1 hybrids exhibited sterility, while female F_1 hybrids continued to lay eggs but showed reduced fertility compared to conspecific crosses in accordance with Haldane's rule (Haldane, 1922). Furthermore, our study revealed strong evidence that intercontinental *S. neocynipsea* populations are indeed the same species because intercrossing resulted in some fitness decrements similar to interspecific crosses, but produced viable and fertile F_1 and, to some extent also F_2 hybrid offspring.

Baseline comparison of the two species

Our study revealed insignificant differences in fecundity and fertility traits between *S. cynipsea* and *S. neocynipsea*, as one could expect due to the similarities in their

ecology and their relatively low heterospecific genetic differentiation (Pont & Meier 2002; Su, Kutty, & Meier, 2008, Baur *et al.*, 2017, Chapter 1). However, the species exhibit significant differences in body size, with *S. neocynipsea* being bigger than *S. cynipsea*, in accordance with previous studies (Puniamoorthy, Schäfer, & Blanckenhorn, 2012; Rohner, Blanckenhorn, & Puniamoorthy, 2016). All other fecundity and fertility traits showed few differences, especially when the body size differences were controlled for, which contrasts with the more pronounced but nevertheless merely quantitative (rather than qualitative) differences in mating behaviour between the two species (Giesen, Blanckenhorn, & Schäfer, 2017, Chapter 2). Roughly two thirds of all females of both species laid their first clutch containing ca. 45 eggs after six days. Thus, a certain proportion of all females was not able to produce any eggs and therefore might be sterile, again in accordance with previous studies of *S. cynipsea* (Blanckenhorn *et al.*, 2002; Teuschl & Blanckenhorn, 2007). This might well be a consequence of our protracted laboratory breeding, as freshly caught field females typically have lower infertility rates (~10 – 20% max; Rohner, pers. comm.). Egg-to-adult survival was also low (~30%), which again might be explained by our samples stemming from long-term laboratory isofemale-lines. Regardless, this low baseline fertility of our iso-female lines ultimately did not impede our ability to detect differences between con- and heterospecific crosses in the fecundity traits investigated, our main goal here, as these were generally substantial.

Heterospecific crosses resulting in F_1 hybrid offspring

As expected, fertility in heterospecific crosses was strongly reduced to roughly 3.5% in both hybridization directions (CN vs. NC), documenting clear mating and fertilization incompatibilities between the species (Turelli, Barton, & Coyne, 2001). This reduction of F_1 viability and fecundity is a typical hybridization pattern evident in several taxa (see Dowling & Secor, 1997; Fitzpatrick, Fordyce, & Gavrilets, 2009), and confirms the assumption of intrinsic postcopulatory incompatibilities between the two species such as disrupted sperm transfer (Arthur & Dyer, 2015), cryptic female discrimination of heterospecific sperm (Eberhard, 1991), or high mortality of hybrid offspring in the stages following zygote formation (Dowling & Secor, 1997; Mayr, 1942). Despite low fertility, egg numbers were similar in heterospecific and conspecific crosses, although the probability to produce eggs was significantly lower for the CN (41%) relative to the NC hybridization direction (64%) and the conspecific

crosses (68%). This could indicate a disruption of egg production by a heterospecific mate due to postmating, prezygotic barriers, for instance due to incompatibilities between sperm and egg surface proteins preventing fertilization (Palumbi, 1999) or toxicity of *S. neocynipsea* sperm for *S. cynipsea* eggs (Rice, 1996). Moreover, the probability of producing eggs in the CN direction decreased towards zero in populations from sympatric areas relative to para- and allopatric ones. This again supports our conjecture about heterospecific sperm possibly disrupting fertilization, which in sympatry might be reinforced by natural selection due to the continuous contact between the two species (Coyne & Orr, 1989, 2004; Turelli, Barton, & Orr, 2001; Yukilevich, 2012), as we found also for some behavioural traits (Giesen, Blanckenhorn, & Schäfer, 2017, Chapter 2).

Reduced hybrid fitness across generations with F_2 hybrid breakdown

Similar to the results of the heterospecific crosses, intercrossed F_1 and F_2 hybrids had very low egg-to-adult viability. Although the body size and egg production of F_1 hybrid intercrosses were not decreased but in fact somewhat higher than those of the heterospecific crosses, indicating hybrid vigour (Todesco *et al.*, 2016; Wolf, Takebayasi, & Rieseberg, 2001), eggs and offspring production was significantly reduced in intercrosses of F_2 hybrids, demonstrating strong hybrid breakdown. Expectable, emergence of F_2 and F_3 hybrid offspring approached zero, demonstrating fertilization difficulties similar to heterospecific crosses (Turelli, Barton, & Orr, 2001; Dowling & Secor, 1997; Palumbi, 1999; Rice, 1996). This mechanism again might show a reinforcement pattern, as sympatric crosses resulted in no offspring at all, while some offspring resulted in parapatric crosses. However, no offspring were produced in allopatric crosses, and the overall sample sizes were low at this stage due to intrinsic incompatibilities between the species.

More interestingly, our data indicate an asymmetry in hybridization direction (CN vs. NC) affecting offspring number and hence fertility of the F_1 generation. If the mating partners resulted from crossing *S. cynipsea* females and *S. neocynipsea* males, they were not able to produce any adult F_2 offspring, similar to the asymmetry regarding egg production in the parental generation. As the less abundant species is more likely to mate heterospecifically (Matute, 2014), *S. neocynipsea* females have a better chance to mate and produce fertile F_1 hybrid offspring with *S. cynipsea* males than *vice versa* (Matsuda *et al.*, 2009; Sawamura *et al.*, 2016). This asymmetry was

still evident in intercrosses of the F_2 hybrids, and explains why we have low sample sizes for the later hybrid generations, as it was very hard to obtain F_2 and F_3 hybrid offspring.

Haldane's rule – male sterility in F_1 hybrid offspring

Our study highlights a breakdown of hybrid offspring production between *S. cynipsea* and *S. neocynipsea*, primarily via a <4% decrease in egg-to-adult viability but also egg production (Fig. 1), signifying strong but not absolute suppression of hybridization. In addition, hybrids performed differently according to sex as predicted by Haldane's rule, according to which the heterogametic sex, here the male, is mostly sterile (Haldane, 1922). This was already visible in the first hybrid generation, but to further examine these deleterious differences we conducted backcrosses between F_1 hybrid offspring with the parental species.

Egg-to-adult viability as well as egg and offspring numbers revealed a certain degree of sterility when hybrid males were forced to mate with *S. cynipsea* or *S. neocynipsea* females, whereas female hybrids showed little to no such decrease in the converse situation. Egg number was also decreased, probably due to sperm incompatibilities or toxicity, as discussed above (Palumbi, 1999; Rice, 1996). These heterogametic problems arise from genetic incompatibilities between hybrids and parental species. In contrast, hybrid females performed equally well in terms of number of eggs produced, clearly demonstrating hybrid female fertility (Fig. 1b). These results agree well with Haldane's rule as found in several other taxa (Dowling & Secor, 1997). However, while fitness of the heterogametic sex is almost suppressed via genetic incompatibilities, the homogametic sex sometimes can also show decreased fitness (e.g. flycatchers: Alatalo *et al.*, 1990; Qvarnström *et al.*, 2016). In this bird example, natural selection acted mainly via sterility of the heterogametic sex (here females), while reduced fitness of the homogametic sex (here males) was driven by sexual selection (Qvarnström *et al.*, 2016). We can conclude that natural selection is the main force driving selection on hybrids via reduced survival, with sexual selection as an additional pre-copulatory force as demonstrated by Giesen, Blanckenhorn, & Schäfer (2017, Chapter 3). Our next step should be determining the extent of hybridization and its underlying evolutionary forces in nature using now available genomic tools (Giesen *et al.*, 2017, Chapter 4).

Cross-continental S. neocynipsea hybridization

We here also explored differences between continental *S. neocynipsea* populations. Although European and North American populations will never come into contact in nature due to their allopatric distribution, they are recognized as the same species based on their similar morphology and behaviour (Pont & Meier, 2002; Giesen, Blanckenhorn, & Schäfer, 2017, Chapter 2). However, population genetic differentiation between the continents is fairly high (F_{ST} = 0.10, Baur *et al.*, 2017, Chapter 1), indicating that potentially random allopatric speciation by genetic drift might be ongoing. There was some evidence for hybridization barriers (Fig. 3) similar to those between the species (Fig. 1). North American *S. neocynipsea* required longer time than European flies to lay their first clutch. This might merely reflect ecological differences due to geography, as body size was controlled for. Egg production and egg-to-adult viability were lower in crosses of North American females with European males (Fig. 3). Therefore, sperm from cross-continental males appears to disturb the fertilization in some way (Arthur & Dyer, 2015), and/or some intrinsic incompatibilities are evolving at least in one direction. Intercrossing of F_2 offspring showed signs of hybrid breakdown too, but this was based only on very low sample sizes.

In contrast to the heterospecific situation in Fig. 1, backcrosses between hybrid offspring and parental species showed no signs of reduced fitness in terms of egg-to-adult viability, such that both hybrid sexes appear to be equally reproductively fertile and genetic incompatibilities according to Haldane's rule have no effect. Overall, our cross-continental *S. neocynipsea* crosses indicate some degree of genetic differentiation, presumably due to genetic drift alone, with associated incompatibilities and fitness decrements following hybridisation, although flies from both continents still recognize each other as the same species (Giesen, Blanckenhorn, & Schäfer, 2017, Chapter 2).

CONCLUSION

This laboratory study revealed strong but not ultimate postzygotic barriers between the closely related sister species *S. cynipsea* and *S. neocynipsea*, with some indication of similar isolating barriers developing among continental European and North American (i.e. allopatric) *S. neocynipsea* populations. As expected, fecundity and viability were significantly decreased in heterospecific compared to their conspecific

parental crosses due to yet unspecified genetic incompatibilities. Furthermore, we detected strong hybrid breakdown in the F_1, F_2, and F_3 generations across several fecundity and fertility traits revealing difficulties to form hybrid offspring, perhaps due to barriers in sperm transfer, difficulties to form a zygote, and/or a decreased viability of the juvenile stages. These showed a hybridization asymmetry with viable female fertility and hybrid offspring but hybrid male (the heterogametic sex) sterility in accordance with Haldane's rule. We conclude that hybridisation between the two species in European areas of sympatry (e.g. the Swiss Alps) is prevented to some degree by reinforcement due to natural selection, whereas the lower fitness of naturally not occurring continental (allopatric) *S. neocynipsea* hybrids is presumably explained by random processes (i.e. genetic drift). Whether there are genomic signs of hybridization in natural populations remains to be investigated (Giesen *et al.*, 2017, Chapter 4).

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Figure 1. Probability of producing eggs (p(eggs)) and offspring (p(offspring)), number of eggs and offspring, as well as egg-to-adult viability for con- and heterospecific crosses between *S. cynipsea* and *S. neocynipsea* in parental, F_1 and F_2 generation hybrids, as well as backcrosses between parental species and F_1 hybrid offspring.

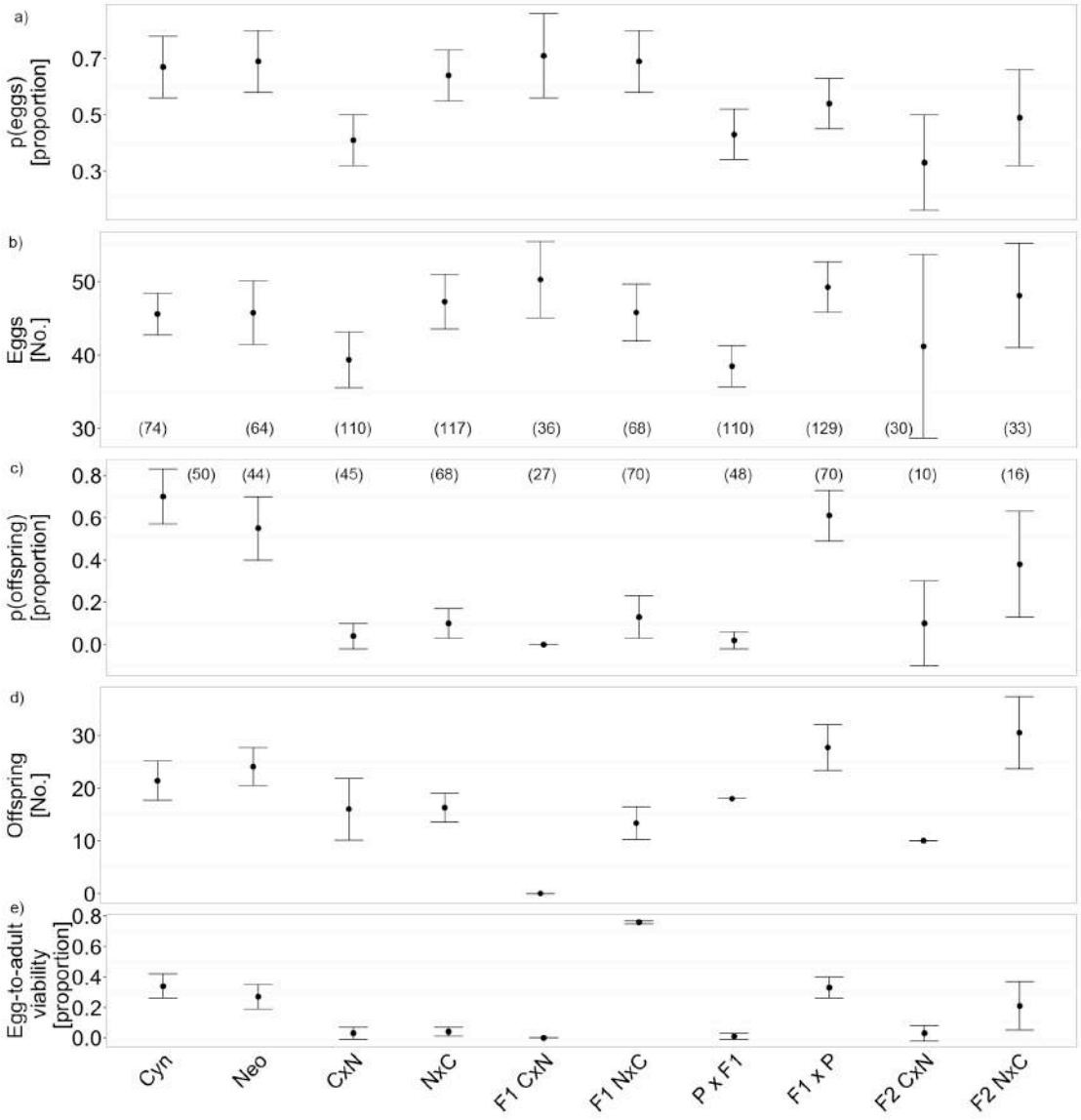

Figure 2. Female age of first reproduction [days], female and male head width [mm] for con- and heterospecific crosses between *S. cynipsea* and *S. neocynipsea* in parental, F_1 and F_2 generation hybrids, as well as backcrosses between parental species and F_1 hybrid offspring.

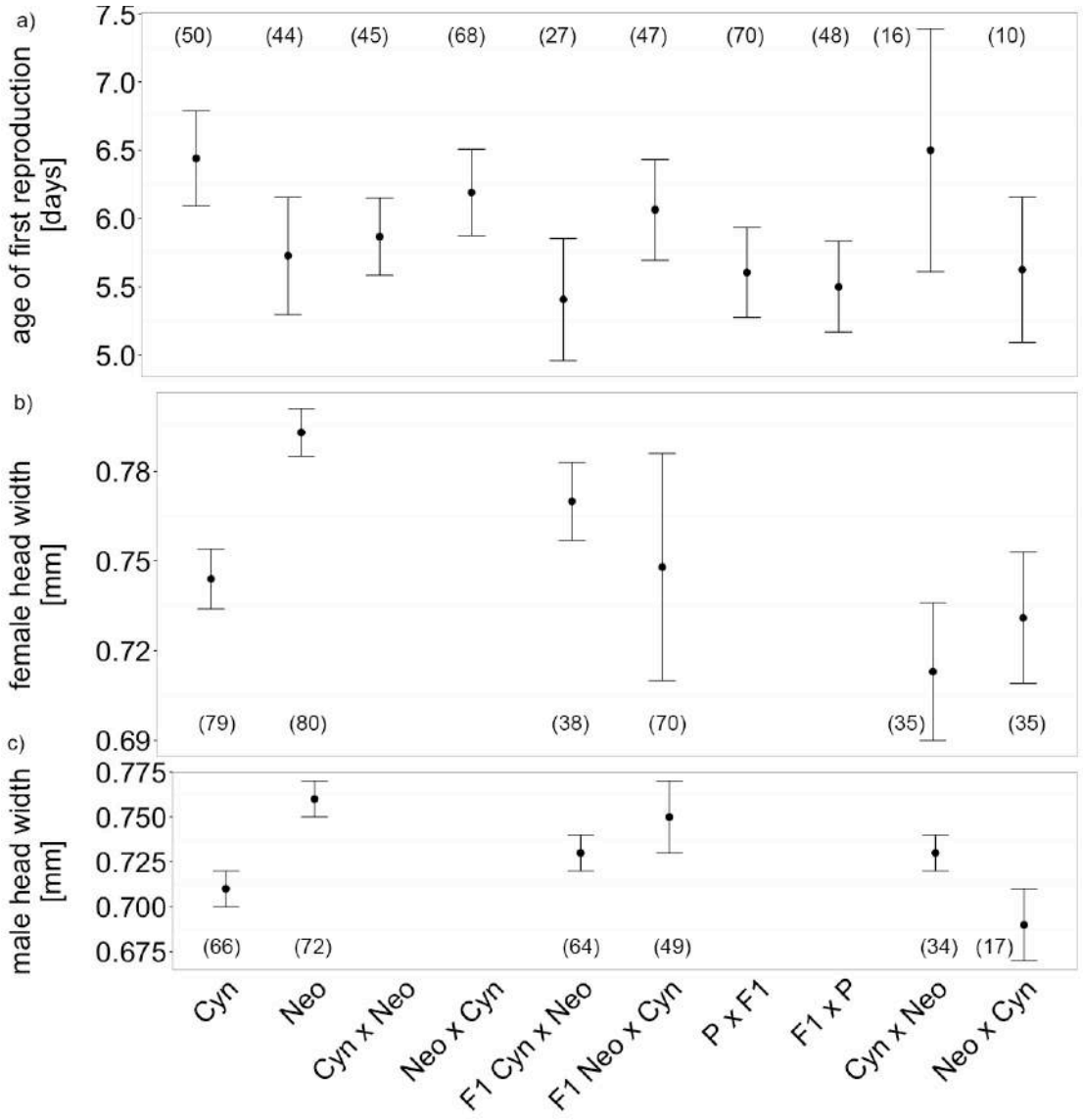

Figure 3. Probability of producing eggs (p(eggs)) and offspring (p(offspring)), number of eggs and offspring, as well as egg-to-adult viability for cross-continental and within population crosses of *S. neocynipsea* populations in parental, F_1 and F_2 generation hybrids, as well as backcrosses between parental species and F_1 hybrid offspring.

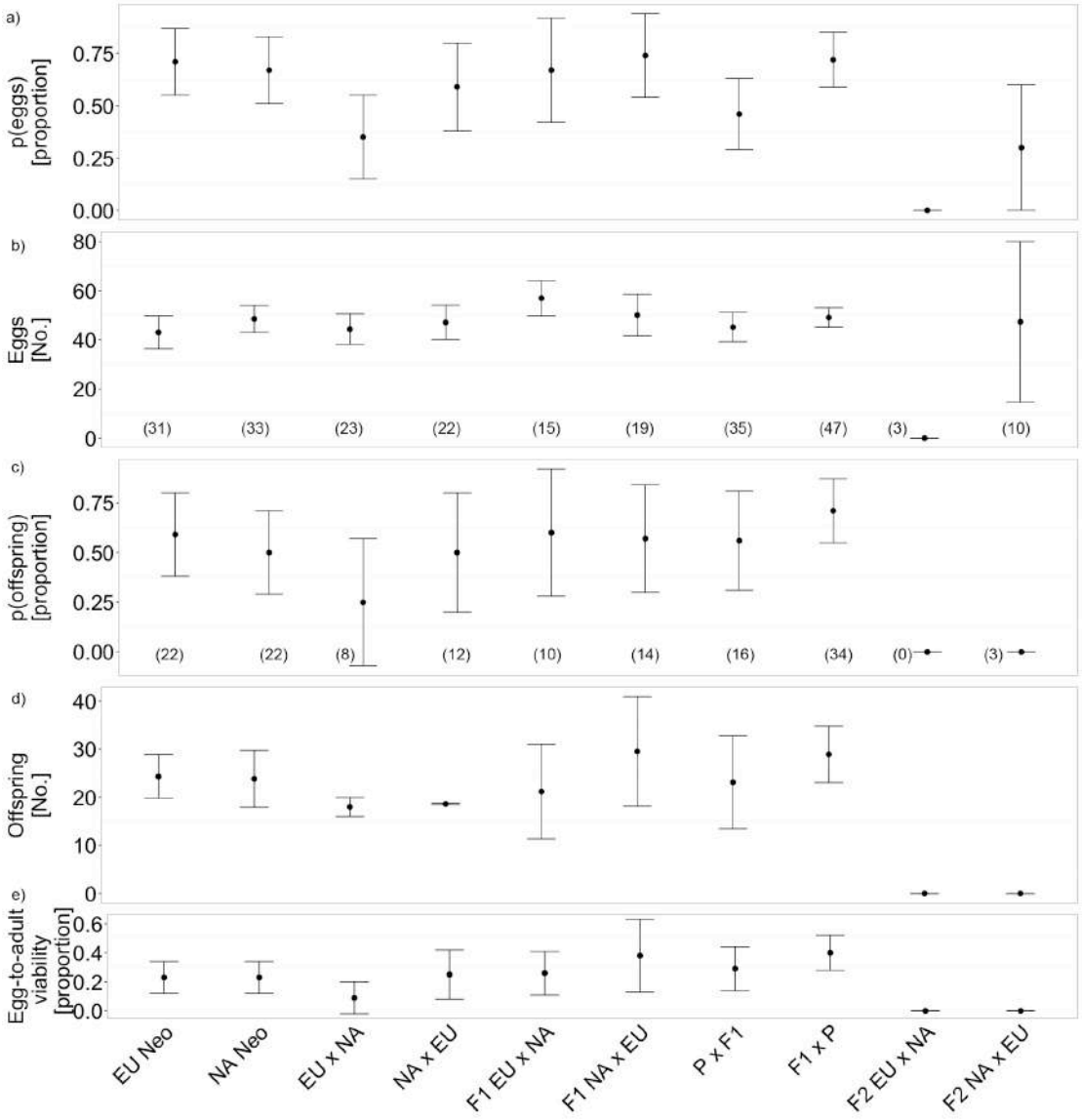

Table 1. Inter-specific (three groups) and intra-specific (one group) crossing scheme of three biogeographical types (female x male). All crosses were reciprocal.

Biogeographical type	Crosses	Population replicates
Sympatry in Europe	EU *S. neocynipsea* x EU *S. cynipsea*	(CH1) x (CH1)
		(CH2) x (CH2)
Parapatry across Europe	EU *S. neocynipsea* x EU *S. cynipsea*	(EU1) x (CH1)
		(EU2) x (CH2)
Allopatry across continents:		
heterospecific	NA *S. neocynipsea* x EU *S. cynipsea*	(NA1) x (CH1)
		(NA2) x (CH2)
conspecific	NA x EU *S. neocynipsea*	(NA1) x (CH1)
		(NA2) x (CH2)

Table 2. Mean egg-to-adult viability, no. and probability of eggs and offspring produced, age at first reproduction, and male and female head width (± 95% CI, female x male, C: *S. cynipsea*, N: *S. neocynipsea*) in parental, F_1 and F_2 hybrid, and backcross pairings between F_1 hybrids and parental species.

Generation	Cross	Egg-to-adult viability	p(eggs) (egg no.)	p(offspring) (offspring no.)	Female head width [mm]	Male head width [mm]	Age First Reproduction [days]
Parental	C	0.34±0.08	0.67±0.11 (45.62±2.87)	0.70±0.13 (21.42±3.74)	0.74±0.01	0.71±0.01	6.44±0.35
	C x N	0.03±0.04	0.41±0.09 (39.36±3.80)	0.04±0.06 (16.00±5.88)	na	na	5.87±0.28
	N x C	0.04±0.03	0.64±0.09 (47.29±3.72)	0.10±0.07 (16.29±2.70)	na	na	6.19±0.32
	N	0.27±0.08	0.69±0.11 (45.77±4.36)	0.55±0.15 (24.08±3.58)	0.79±0.01	0.76±0.01	5.73±0.43
	N_{EU}	0.23±0.11	0.71±0.16 (43.05±6.66)	0.59±0.21 (24.31±4.52)	0.79±0.01	0.76 ± 0.01	4.91±0.18

Table 2. cont.

Generation	Cross	Egg-to-adult Viability	p(eggs) (Egg No.)	p(offspring) (Offspring no.)	Female head width [mm]	Male head width [mm]	Age First Reproduction [days]
Parental	N_{EU} x N_{NA}	0.09±0.11	0.35±0.20 (44.31±6.19)	0.25±0.32 (18.00±1.96)	na	na	5.25±0.89
	N_{NA} x N_{EU}	0.25±0.17	0.59±0.21 (47.08±7.05)	0.50±0.30 (18.59±0.21)	na	na	5.15±0.54
	N_{NA}	0.23±0.11	0.67±0.16 (48.50±5.55)	0.50±0.21 (23.82±5.93)	0.79±0.01	0.77±0.01	6.55±0.69
	conspecific	0.30±0.06	0.69±0.08 (45.69±2.52)	0.63±0.10 (22.86±2.60)	na	na	6.12±0.28
	heterospecific	0.04±0.03	0.53±0.07 (44.13±2.79)	0.08±0.05 (16.56±2.68)	na	na	6.06±0.22

Table 2. cont.

Generation	Cross	Egg-to-adult Viability	p(eggs) (Egg No.)	p(offspring) (Offspring no.)	Female head width [mm]	Male head width [mm]	Age First Reproduction [days]
F_1 hybrids	C x N	0.00±0.00	0.71±0.15 (50.30±5.22)	0.00±0.00 (0.00±0.00)	0.77±0.01	0.73±0.01	5.41±0.45
	N x C	0.76±0.01	0.69±0.11 (45.81±3.89)	0.13±0.10 (13.33±3.11)	0.75±0.04	0.75±0.02	6.06±0.37
	N_{EU} x N_{NA}	0.26±0.15	0.67±0.25 (56.90±7.17)	0.60±0.32 (21.17±9.84)	0.77±0.03	na	5.60±0.98
	N_{NA} x N_{EU}	0.38±0.25	0.74±0.20 (50.05±8.42)	0.57±0.27 (29.50±11.38)	0.73±0.03	na	6.64±0.67
F_2 hybrids	C x N	0.03±0.05	0.33±0.17 (41.20±12.53)	0.10±0.20 (10.00±0.00)	0.71±0.02	0.73 ± 0.01	6.50±0.89

Table 2. cont.

Generation	Cross	Egg-to-adult Viability	p(eggs) (Egg No.)	p(offspring) (Offspring no.)	Female head width [mm]	Male head width [mm]	Age First Reproduction [days]
F_2 hybrids	N x C	0.21±0.16	0.49±0.17 (48.13±7.11)	0.38±0.25 (30.50±6.84)	0.73±0.02	0.69 ± 0.02	5.63±0.53
	N_{EU} x N_{NA}	0.00±0.00	0.00±0.00 (0.00±0.00)	0.00±0.00 (0.00±0.00)	6.24±0.06	na	na
	N_{NA} x N_{EU}	0.00±0.00	0.30±0.30 (47.33±32.73)	0.00±0.00 (0.00±0.00)	0.71±0.05	na	5.00±1.96
Backcrosses	P x F_1 (hetero)	0.01±0.02	0.43±0.09 (38.48±2.80)	0.02±0.04 (18.00±0.00)	na	na	5.60±0.33
	F_1 x P (hetero)	0.33±0.07	0.54±0.09 (49.27±3.44)	0.61±0.12 (27.72±4.34)	na	na	5.50±0.34
	P x F_1 (cont.)	0.29±0.15	0.46±0.17 (45.19±5.92)	0.56±0.25 (23.11±9.63)	na	na	5.38±0.40
	F_1 x P (cont.)	0.40±0.12	0.72±0.13 (49.06±3.96)	0.71±0.16 (28.92±5.87)	na	na	5.71±0.38

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APPENDIX

Table A1. Univariate analyses of variance comparing con- and heterospecific parental crosses for all reproductive traits (cross-continental *S. neocynipsea* crosses in a separate analysis). No covariates were significant.

Pairing	Comparison	Trait	*df*	X^2	*p*	*β*
Parental	C, N	Egg to adult viability	1, 6	1.410	0.235	
		No. eggs	1, 6	1.338	0.247	
		p(eggs)	1, 6	0.038	0.846	
		No. offspring	1, 6	0.371	0.542	
		p(offspring)	1, 6	3.866	**0.049**	
		Reproductive age	1, 6	2.037	0.154	
		Female body size	1, 6	6.668	**<0.001**	
		Male body size	1, 5	7.771	**0.005**	
	CN, NC	Egg to adult viability	1, 6	1.855	0.173	
		No. eggs	1, 6	0.316	0.574	
		p(eggs)	1, 6	8.049	**0.005**	
		No. offspring	1, 1	2.764	0.096	
		p(offspring)	1, 6	0.999	**<0.001**	
		Reproductive age	1, 6	2.130	0.144	

Table A2. Univariate analyses of variance comparing hybridization direction of F1 and F2 hybrid crosses for all reproductive traits (cross-continental *S. neocynipsea* crosses in a separate analysis). No covariates were significant. Lower degrees of freedom resulted from some crosses having no reproductive success at all.

Pairing	Comparison	Trait	*df*	X^2	*p*	*β*
F1 hybrids	CN, NC	Egg to adult viability*	na			
		No. eggs	1, 4	0.767	0.381	
		p(eggs)	1, 4	0.000	1.000	
		No. offspring	1, 2	5.099	**0.024**	
		p(offspring)*	na			
		Reproductive age	1, 4	0.406	0.524	
		Female body size	1, 4	4.906	**0.027**	
		Male body size	1, 4	6.560	**0.010**	
F2 hybrids	CN, NC	Egg to adult viability*	na			
		No. eggs	1, 3	4.467	**0.035**	
		p(eggs)	1, 4	0.001	0.999	
		No. offspring	1, 2	7.582	**0.006**	
		p(offspring)*	na			
		Reproductive age	1, 3	2.101	0.147	
		Female body size	1, 4	0.537	0.464	
		Male body size	1, 1	1.190	0.275	

* no statistics applicable due to lack of emerged flies

Table A3. Univariate analyses of variance comparing hybridization direction of backcrosses between F1 hybrid offspring and their parental species for all reproductive traits (cross-continental *S. neocynipsea* crosses in a separate analysis). Only significant covariates are reported. Lower degrees of freedom resulted from some crosses having no reproductive success at all.

Pairing	Comparison	Trait	*df*	X^2	*p*	*β*
Backcrosses for	F1xP, PxF1	Egg to adult viability	1, 12	67.372	**<0.001**	
heterospecific		No. eggs	1, 12	16.570	**<0.001**	
crosses		p(eggs)	1, 20	0.001	0.999	
		No. offspring	1, 8	1.387	0.239	
		p(offspring)	1, 8	1.387	0.239	
		Reproductive age	1, 12	0.542	0.462	
Backcrosses for	F1xP, PxF1	Egg to adult viability	1, 6	2.894	0.089	
cross-continental		No. eggs	1, 6	1.063	0.303	
S. neocynipsea		p(eggs)	1, 6	0.001	0.999	
		No. offspring	1, 4	1.342	0.247	
		p(offspring)	1, 6	0.001	0.999	
		Reproductive age	1, 6	2.792	0.095	

Table A4. Univariate analyses of variance comparing con- vs. heterospecific parental and F1 crosses for all reproductive traits (cross-continental *S. neocynipsea* crosses excluded). No covariates were significant. Lower degrees of freedom resulted from some crosses having no reproductive success at all.

Pairing	Comparison	Trait	*df*	X^2	*p*	*β*
Parental	con-, heterospecific	Egg to adult viability	1, 15	50.704	**<0.001**	
		No. eggs	1, 15	1.338	0.247	
		p(eggs)	1, 19	2.928	0.087	
		No. offspring	1, 9	0.978	0.323	
		p(offspring)*	na			
		Reproductive age	1, 15	0.835	0.361	
F1 vs. Parental	F1, conspecific	Egg to adult viability	1, 10	30.635	**<0.001**	
		No. eggs	1, 10	0.430	0.512	
		p(eggs)	1, 12	0.001	0.999	
		No. offspring	1, 6	1.564	0.211	
		p(offspring)	1, 10	0.001	0.999	
		Reproductive age	1, 10	0.928	0.335	
		Female body size	1, 10	0.424	0.515	
		Male body size	1. 8	9.163	**0.002**	

* no statistics applicable due to a lack of emerged flies

Table A5. Univariate analyses of variance comparing conspecific parental and F2 crosses for all reproductive traits (cross-continental *S. neocynipsea* crosses excluded). No covariates were significant.

Pairing	Comparison	Trait	*df*	X^2	*p*	*β*
F2 vs. Parental	F2, conspecific	Egg to adult viability	1, 6	4.996	**0.025**	
		No. eggs	1, 6	0.327	0.567	
		p(eggs)	1, 6	0.001	0.998	
		No. offspring	1, 6	0.001	0.975	
		p(offspring)	1, 6	0.001	0.998	
		Reproductive age	1, 6	0.366	0.545	
		Female body size	1, 6	60.111	**<0.001**	
		Male body size	1, 6	33.873	**<0.001**	

Table A6. Univariate analyses of variance comparing parental continental and cross-continental crosses of *S. neocynipsea* for all reproductive traits. No covariates were significant. Lower degrees of freedom resulted from some crosses having no reproductive success at all.

Pairing	Comparison	Trait	*df*	X^2	*p*	*β*
Parental	EU, NA	Egg to adult viability	1, 2	1.098	0.295	
S. neocynipsea		No. eggs	1, 2	0.820	0.365	
		p(eggs)	1, 2	0.211	0.646	
		No. offspring	1, 2	0.146	0.702	
		p(offspring)	1, 2	0.596	0.440	
		Reproductive age	1, 2	26.514	**<0.001**	
		Female body size	1, 2	0.001	0.999	
		Male body size	1, 1	8.017	**0.005**	
Parental	EUxNA, NAxEU	Egg to adult viability	1, 2	3.781	0.052	
cross-continental		No. eggs	1, 2	0.165	0.685	
S. neocynipsea		p(eggs)	1, 2	3.133	**0.007**	
		No. offspring	1, 1	0.094	0.759	
		p(offspring)	1, 2	0.001	0.999	
		Reproductive age	1, 2	1.423	0.233	

Table A7. Univariate analyses of variance comparing hybridization direction of F1 continental *S. neocynipsea* crosses, as well as F1 hybrids with their parental continental parental populations for all reproductive traits. No covariates were significant. Lower degrees of freedom resulted from some crosses having no reproductive success at all.

Pairing	Comparison	Trait	*df*	X^2	*p*	*β*
F1	EU, NA	Egg to adult viability	1, 2	0.003	0.959	
S. neocynipsea		No. eggs	1, 2	3.012	0.083	
		p(eggs)	1, 2	2.285	0.131	
		No. offspring	1, 2	0.389	0.533	
		p(offspring)	1, 2	0.001	0.999	
		Reproductive age	1, 2	8.327	**0.004**	
F1 vs Parental	F1, continental	Egg to adult viability	1, 4	2.221	0.136	
S. neocynipsea		No. eggs	1, 4	7.210	**0.007**	
		p(eggs)	1, 4	0.008	0.929	
		No. offspring	1, 4	0.859	0.354	
		p(offspring)	1, 4	0.001	0.999	
		Reproductive age	1, 4	0.954	0.329	

Hybridization and introgression at the genomic level between the sister species *Sepsis cynipsea* and *S. neocynipsea* (Diptera: Sepsidae) in Swiss regions of sympatry

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ABSTRACT

Recent research indicates that hybridisation not only has deleterious fitness effects, but may also fuel adaptive diversification and speciation by introgression of beneficial alleles. Using whole genome sequences (scaffolds) of four iso-female lines originating from the Swiss lowlands (Zürich) and the Swiss Alps (Sörenberg), we tested for genome-wide patterns of introgression between the closely related dung fly species *Sepsis cynipsea* and *S. neocynipsea* (Diptera: Sepsidae). Previous microsatellite results and laboratory hybridisation experiments showed that these sister species are genetically distinct but may hybridize in nature in areas of sympatry. We thus tested for introgression, using *S. orthocnemis* as an outgroup, with a phylogenomic approach using ABBA-BABA-statistics. The data indicate bidirectional introgression between the species in Zürich but not the Swiss Alps. We further detected signatures of unidirectional introgression from alpine *S. neocynipsea* into lowland *S. cynipsea* and from Alpine *S. cynipsea* into lowland *S. neocynipsea*, although some tests were only marginally significant. This pattern was supported by corresponding preliminary analyses of pooled population sequences. Relatively weak signatures of introgression combined with rather few and small introgressed regions suggest this gene exchange is ancient with most regions by now homogenized by recombination and/or selection. Apparently, premating behavioral barriers and postmating fertility reductions, in conjunction with micro-ecological niche differences mediating spatio-temporal divergence in reproductive timing, effectively prevent present hybridization in nature. Work in progress will provide further insights into patterns of introgression across the species' natural ranges as well as the role of latitudinal adaptation in shaping genome-wide patterns of genetic variation.

INTRODUCTION

According to the biological species concept, species represent a group of individuals that are reproductively isolated from other groups. The advent of new species was historically explained by a common ancestor gradually splitting into typically two reproductively isolated lineages (Coyne & Orr, 2004; Dobzhansky, 1951; Mayr, 1963). First botanists and later zoologists described hybridization beyond species boundaries occurring regularly in various taxa in nature (Anderson, 1949; Barton & Bengtsson, 1986; cf. DeMarais *et al.*, 1992; Gante *et al.*, 2016; Mallet, 2007; Nolte & Tautz, 2010; Rieseberg *et al.*, 2003; Trier *et al*., 2014). Research over

the last decades indicates that hybridization not only has deleterious effects due to negative epistatic interactions within admixed genomes, but can also fuel adaptive diversification and speciation by introgression of beneficial alleles that have already been exposed to selection in one of the parental species (Arnold & Meyer, 2006; Berner & Salzburger, 2015; Seehausen, 2004; Saetre, 2013). Thus, patterns of genomic divergence among recently formed species can be viewed as a patchwork of genomic regions with significant differentiation punctuated by more freely introgressing blocks of DNA (Nosil *et al.*, 2009; Wu, 2001). Such blocks can be identified by genome scans as long as these blocks are still in linkage disequilibrium with genomic regions encoding for traits engaged in adaptation and reproductive isolation (Via & West, 2008; Wood *et al.*, 2008).

Sepsid flies (Diptera: Seaside) have served as model organisms in various studies of sexual selection, ecological adaptation, and speciation (Blanckenhorn *et al.*, 1999, 2000; Eberhard, 1999; Kraushaar & Blanckenhorn, 2002; Parker, 1972a,b; Puniamoorthy *et al.*, 2009; Pont & Meier, 2002; Ward, 1983; Ward, Hemmi, & Rösli, 1992). In particular, closely related species pairs, such as *Sepsis cynipsea* and *S. neocynipsea*, provide unique opportunities to address the genomic consequences of hybridization and introgression during early stages of speciation. These two widespread species occur in sympatry across parts of their natural range. Whereas *S. cynipsea* is the most abundant sepsid species in north-central Europe, *S. neocynipsea* is common throughout North America, where it essentially occupies the ecological niche that *S. cynipsea* has in Europe. However, while overall extremely rare in European lowlands, *S. neocynipsea* can be locally common at high altitude sites, such as the Swiss Alps, where it co-occurs in sympatry with *S. cynipsea* (Ozerov, 2005; Pont & Meier, 2002; Rohner, Blanckenhorn, & Puniamoorthy, 2015). Although the species are genetically distinct, they also share significant variation in morphology and behavior (Baur *et al.*, 2017, Chapter 1; Giesen *et al.*, 2017, Chapters 2, 3). Previous studies demonstrated successful hybridization under laboratory conditions with strong but not ultimate pre- and postmating isolating barriers that are partly due to reinforcement in areas of sympatry (Giesen *et al.*, 2017, Chapter 2, Chapter 3). This suggests some amount of gene flow between the sister species. To address the extent of introgression at the genome-wide scale, we sequenced single iso-female lines of both species collected in the Swiss lowland (Zürich, ~450 m above sea level) and the Swiss Alps (Sörenberg, ~1200 m above sea level). These locations are geographically

approximately 70 km linear distance from each other with no major physical barrier in between preventing gene flow among species and populations. Based on Single Nucleotide Polymorphisms (SNPs) we conducted phylogenomic tests for gene flow with ABBA-BABA- and D-statistics (Green *et al.*, 2010; Durand *et al.*, 2011), using all possible triplets and *S. orthocnemis* as an outgroup. Our results indicate bidirectional introgression in lowland Zürich but not the Swiss Alps. We further detected signatures of unidirectional introgression from alpine *S neocynipsea* into lowland *S. cynipsea* and from alpine *S. cynipsea* into lowland *S. neocynipsea*. These patterns are supported by preliminary analyses of corresponding pooled population sequence data (see Appendix I).

MATERIALS & METHODS

Fly origin and culturing

Gravid females of *Sepsis cynipsea* and *S. neocynipsea* were collected from two locations in the Swiss Alps (Sörenberg: 46°24'8.24''N 8°21'28.43''E) and the Swiss lowlands (Zürich; 47°24'0.60''N 8°34'23.97''E) roughly 70 km distant from each other. While both species are abundant in Sörenberg, *S. cynipsea* is the dominant species around Zürich. Laboratory culturing conditions of iso-female lines are described in detail in Giesen *et al.* (2017, Chapter 2).

To obtain sufficient amounts of DNA for our phylogenomic analysis we pooled 50 males of a randomly chosen iso-female line representing descendants of a single wild caught female that had been propagated in the laboratory for numerous generations prior to our study. Use of single highly inbred iso-female lines in this context minimizes false positives in introgression tests that might arise from allelic variation within populations. Genomic DNA was extracted using UltraPure Phenol:Chloroform:Isoamyl alcohol (25:24:1, v/v, Thermo Fischer Scientific) according to the manufacturer's protocol. Quantification of gDNA was performed with a Qubit Fluorometric Quantitation (Thermo Fischer Scientific, Appendix Table A1).

Sequence qualification and next generation re-sequencing

Library preparation was done with TruSeq DNA PCR-Free Library Preparation (Illumina) kit according to the manufacturer's protocol. This kit represents a PCR-free approach minimizing statistical errors arising from PCR duplicates during

bioinformatic processing. Libraries were validated with TapeStation 2200 (Agilent Technologies, Waldbronn, Germany, Appendix Table A1). Sequencing on Illumina HiSeq 2500 v4 was conducted by pooling the four iso-female lines onto one lane to achieve a ~60x coverage.

Bioinformatic processing, assembly and alignment

Qualitative validation of sequence data before and after trimming was done with FastQC High Troughput Sequence QC Report Version 0.11.4 (Andrews *et al.*, 2011). Sequence adapters specific for Illumina Technologies were removed with Trimmomatic Version 0.36 (Bolger, Lohse, & Usadel, 2014). Preliminary whole genome sequences (scaffolds) of *Sepsis orthocnemis* were used as the outgroup to test for signatures of introgression. Sequenced *S. cynipsea* and *S. neocynipsea* iso-female lines were aligned against *S. orthocnemis* using the Burrows-Wheeler-Aligner Version 0.7.12 (Li & Durbin, 2009). Approximately 65% of the trimmed reads of both species could be mapped to the outgroup reference (Appendix Table A1). File conversions and sorting, SNP calling and filtering (SNP call quality of above 40) were performed in samtools Version 1.3.1 (Li *et al.*, 2009).

Introgression tests and population genetic differentiation

Tests for introgression were conducted with ABBA-BABA-statistics (Green *et al.*, 2010; Durand *et al.*, 2011). In a phylogeny with a topology as depicted in Figure 1, allele B arises via mutation from allele A under stochastic lineage sorting with equal probability of ABBA or BABA patterns. Gene flow between the heterospecific lineages P3 and P2 after the bifurcating speciation process leads to an excess of ABBA patterns, which is validated with Patterson's D-statistics implemented in the ABBA-BABA-statistics (Green *et al.*, 2010; Durand *et al.*, 2011). This phylogenomic method has already successfully been applied to genome wide data from various organisms and sequencing techniques, including whole genome sequencing (Green *et al.*, 2010), RADseq protocols (Eaton & Ree, 2013; Meier *et al.*, 2017; Streicher *et al.*, 2014), and exon capture data (Heliconius Genome Consortium, 2012).

We tested for introgression using four main comparisons of iso-female lines (Table 1; Fig. 2). Each comparison can be rearranged on the phylogenomic tree in three possible combinations, so that all possible combinations (N=12) of the tree were represented (Table 1). In the ideal phylogenomic tree P1 and P2 should always cluster

according to species status with P3 as sister species, which can show gene flow with either P1 or P2. If P3 unidirectional exchanges genes into P2, we expect an excess of ABBA compared to BABA patterns resulting in a positive sign of the Patterson's D-statistic (see Fig. 1; Green *et al.*, 2010; Durand *et al.*, 2011).

To count ABBA-BABA-variants from the mapped data and to estimate D-statistics we used filtered SNPs as input in the program ANGSD Version 0.914 (Korneliussen, Albrechtsen, & Nielsen, 2014). The program calculates genotype likelihood estimates for each DNA block of 5 kb across the genome. To evaluate statistical robustness of our results we tried different block sizes (10kb, 50 kb, 100kb). Since we did not obtain any significant variation between these, we only report the results for the analysis with 5kb blocks. To visualize and detect any regions influenced by introgression, we wrote and ran a script in R (Appendix II Script). ANGSD Version 0.914 was used to calculate pairwise F_{ST}–values between the sequenced iso-female lines.

RESULTS

All introgression tests between iso-female lines are shown in Table 1 (see also Table A1 and Table A2futschi). Uni- and bidirectional gene flow between the species of Zürich and Sörenberg is summarized in Fig. 2.

We detected significant gene flow between Zürich *S. cynipsea* and *S. neocynipsea* in two (as opposed to merely one) tests with Z-scores ranging between -4.074 and -4.266 (Table 1b, d), implying bidirectional introgression. Corresponding skyline plots of the D-statistics show small signatures of significant introgression spread roughly randomly around the genome (Figures 3 & 4). Accordingly, the number of introgressed patterns across the genome detected by the excess of BABA-patterns in both tests differed significantly but only with low numbers from the ABBA-patterns, which identified conspecific gene exchange.

In addition, almost significant unidirectional introgression was found for Zürich *S. cynipsea* receiving genes from Sörenberg *S. neocynipsea* in one test (Table 1a, Z=-3.829). Another test also indicated almost significant unidirectional introgression from Sörenberg *S. cynipsea* to Zürich *S. neocynipsea* (Table 1c, Z=-3.669). Similar to the results of higher BABA- than ABBA-patterns for the tests across species of Zürich (see Table 1b, d), the numbers of exceeding BABA-patterns indicating introgression differ only with low numbers from ABBA-patterns.

Lastly, no signs of introgression were evident between the species from Sörenberg in the Swiss Alps, as no tests showed any gene exchange.

Pairwise F_{ST}-analysis revealed significant genetic differentiation between species as well as between iso-female lines within species. Pairwise F_{ST}-values between the species ranged from 0.611 to 0.628. Population genetic differentiation among populations of one species were much lower but significant (*S. cynipsea*: F_{ST}=0.210, *S. neocynipsea*: F_{ST}=0.270). These F_{ST}-values should largely reflect effects of inbreeding associated with the propagation of iso-female lines since microsatellite data revealed absence of significant population structure over large geographic distances in both species (Chapter 1).

DISCUSSION

This study provides significant insights into the evolutionary history of two incipient species of sepsid flies for which we have detailed information on ecology, morphology and behavior (Blanckenhorn *et al.*, 1999, 2000; Eberhard, 1999; Ozerov, 2005; Parker, 1972a,b; Pont & Meier, 2002; Puniamoorthy *et al.*, 2009; Rohner, Blanckenhorn, & Puniamoorthy, 2016; Ward, 1983; Ward, Hemmi, & Rösli, 1992). Previous results obtained from microsatellite and morphometric analysis (Baur *et al.*, 2017, Chapter 1) as well as laboratory hybridization experiments (Giesen, Blanckenhorn, & Schäfer, 2017, Chapters 2, 3) indicated that *S. cynipsea* and *S. neocynipsea* are genetically distinct but might hybridize in nature. Indeed, by scanning the entire genome of representative iso-females collected in geographic areas of sympatry in Switzerland, we detected signatures of introgression between the lineages after the speciation process.

By employing whole genome sequencing we confirmed that *S. cynipsea* and *S. neocynipsea* form clearly distinct genetic entities, which is in agreement with previous results from our microsatellite analyses showing pronounced differentiation between *S. cynipsea* and New and Old world populations of *S. neocynipsea* (Baur *et al.*, 2017, Chapter 1). Nevertheless, our phylogenomic approach provided clear evidence for introgression between the study species. The strongest evidence for introgression was detected in the Swiss lowlands, where two of our four comparisons were statistically significant (Table 1b,d). The relatively low (negative) Z-scores, in combination with the few and spread genomic regions showing signatures of introgression (Fig. 4, 5), suggest that the genetic exchange between the two species is

probably quite ancient, with sufficient time having passed for natural or sexual selection to eliminate most contiguous parts of the introgressed genome. Similar results and conclusions were obtained from introgression studies of humans indicating that selection has eliminated most Neanderthal alleles in modern humans after ancient hybridization between them (Juric, Aeschbacher, & Coop, 2016). Ancient introgression in sepsid flies is plausible given that both species have similar ecological niches and likely shared habitats (open grasslands featuring large vertebrate excrements) throughout their evolutionary history. However, our forced laboratory hybridization experiments showed that mating between the two species successfully occurs, producing fertile F_1 hybrid females and even offspring in backcrosses with the parental species (Giesen, Blanckenhorn, & Schäfer, 2017, Chapter 3). Thus hybridization in nature could take place even nowadays in areas of sympatric co-occurrence such as Zürich or Sörenberg. Despite this, our introgression results indicated only ancient, but no recent gene exchange. Apparently the often subtle premating behavioral barriers and the (post-mating) fecundity and fertility problems documented by Giesen, Blanckenhorn, & Schäfer (2017, Chapter 2, Chapter 3) of this thesis, in conjunction with micro-ecological niche differences mediating spatio-temporal divergence in reproductive timing (pers. observation), are sufficient to effectively prevent hybridization in nature. This interpretation is strengthened by Puniamoorthy's (2014) study showing that mating between two disjunct Central American populations of *Archisepsis diversiformis* was only evident under forced laboratory conditions, while under conditions of free mate choice flies from the different populations did not hybridize. Behavioral mating barriers thus can evolve rather quickly (Puniamoorthy, 2014), especially in sympatry when reinforcement by natural or sexual selection can operate, as in the case of the sister species investigated here (Giesen, Blanckenhorn, & Schäfer, 2017, Chapter 2).

In theory introgression can be bidirectional or unidirectional depending on species or mate recognition systems and the genetic compatibility between parental genomes. Our phylogenomic analysis provides some evidence for both. Bidirectional gene flow between the study species was detected in the Swiss lowland (Tables 1b,d). This result is consistent with our laboratory hybridization experiments showing that both species mate bidirectionally and produce viable F_1 hybrid offspring with only males being sterile according to Haldane's rule (Haldane, 1922). Similarly, Dafu *et al.* (2016) found that gene flow between two lineages of *Picea brachytyla* trees in the

Qinghai-Tibet Plateau was extensive and bidirectional, which might have contributed to the observed morphological similarity. Although our study sepsids share significant variation in morphology and behavior, the similarities arising from introgression seems unlikely in light that genome-wide signatures of introgression were quite weak. Our introgression tests further indicated weak unidirectional introgression from Sörenberg *S. neocynipsea* into Zürich *S. cynipsea* (Table 1a) as well as from Sörenberg *S. cynipsea* into Zürich *S. neocynipsea* (Table 1c; Fig. 2). Unidirectional introgression between species from the Alpine region into lowland populations seems plausible in light of the evolutionary timescale and the short geographic distance (70 km) between the locations. However, we would have to explain why the opposite introgression is not equally plausible, as both species readily occupy cooler high altitude sites (Rohner, Blanckenhorn, & Schäfer, 2015). Interestingly, corresponding preliminary analyses shown in the Appendix I based on pooled population sequences provide additional support for this unidirectional gene flow from *S. neocynipsea* of the Alps into *S. cynipsea* from Zürich (see Appendix I, Table A2 and Fig. A1). These population based comparisons indicate that the lowland population of *S. cynipsea* has received significant proportions of introgressed alleles from the *S. neocynipsea* population inhabiting the Swiss Alps. However, since the ABBA-BABA-statistics used here was explicitly designed for introgression analyses of either single individuals or highly inbred lines, these results should be interpreted cautiously and are therefore only shown in the supplement.

In conclusion, the present study provides significant evidence for introgression between two closely related species of sepsid flies collected in geographic areas of co-existence. The relatively weak but statistically significant signatures of introgression in combination with the relatively few and small introgressed regions (Fig. 3 & 4) suggest this gene exchange is quite ancient, such that large portions of introgressed regions should have been eliminated by recombination and/or selection. Hybridization between the two incipient species might stopped in nature due to adaptation to different ecological niches, such as different breeding times or temperatures, and/or stronger pre- and postmating reproductive barriers preventing successful copulation and hybrid offspring, leading to two distinct species with high interspecific genetic differentiation similar to our results of Chapter 1. Due to the geographic distribution, sympatric and parapatric populations of both species were most likely to undergo hybridization, while gene exchange between allopatric populations was prevented due

to geographic barriers. Therefore, we only tested here with sympatric populations of both species for introgression in nature on the genomic level. Currently we are analyzing patterns of introgression and genetic differentiation by comparing sym-, para-, and allopatric populations of *S. cynipsea* and *S. neocynipsea* collected from multiple locations across Europe and North America. The forthcoming results should provide further insights into patterns introgression across large parts of the species' natural ranges as well as the role of latitudinal adaptation in shaping genome-wide patterns of differentiation.

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Figure 1. Phylogenomic tree model used in ABBA-BABA-statistics to investigate introgression patterns among triplets of *Sepsis cynipsea* and *S. neocynipsea* from two Swiss sites (Zürich, Sörenberg) in all possible combination; outgroup for alignment and testing was always *S. orthocnemis*.

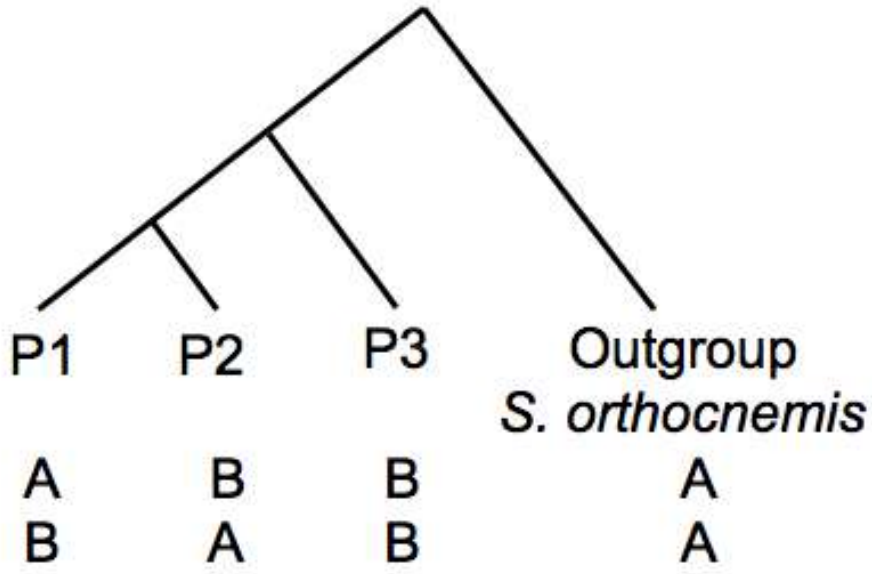


Figure 2. Map of significant gene flow patterns between Swiss lowland (Zürich) and alpine (Sörenberg) populations of *Sepsis cynipsea* and *S neocynipsea*. The width of the arrow indicates the strength of significance (Z-scores).

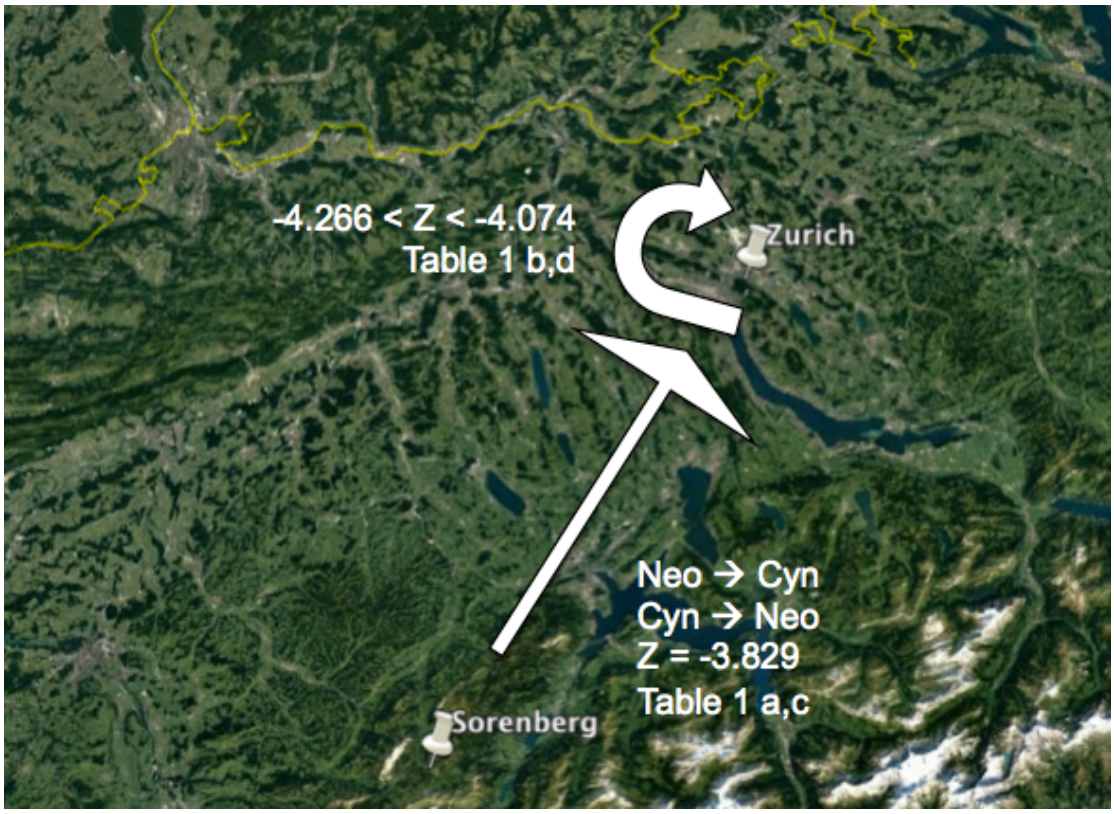


Figure 3. Visualization of D-statistics for introgression patterns across the largest 30 scaffolds. a) Test as shown in Table 1b: P1 – Zürich *S. cynipsea*, P2 – Sörenberg *S. cynipsea*, P3 – Zürich *S. neocynipsea*, b) test as shown in Table 1d: P1 – Zürich *S. neocynipsea*, P2 – Sörenberg *S. neocynipsea*, P3 – Zürich *S. cynipsea*.

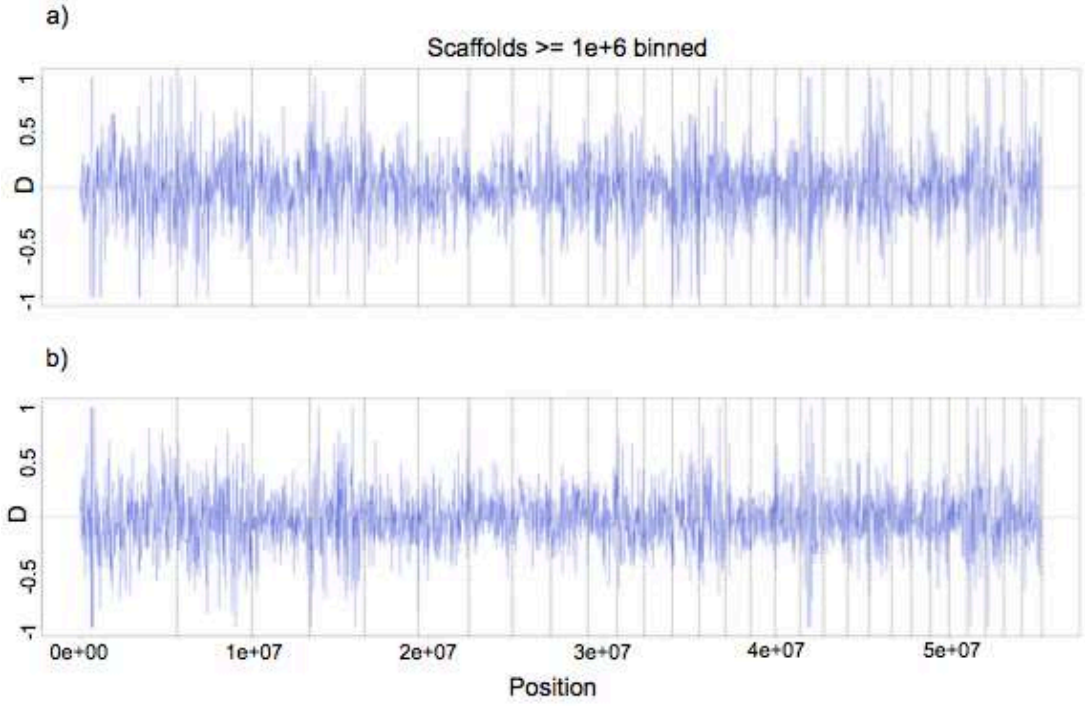
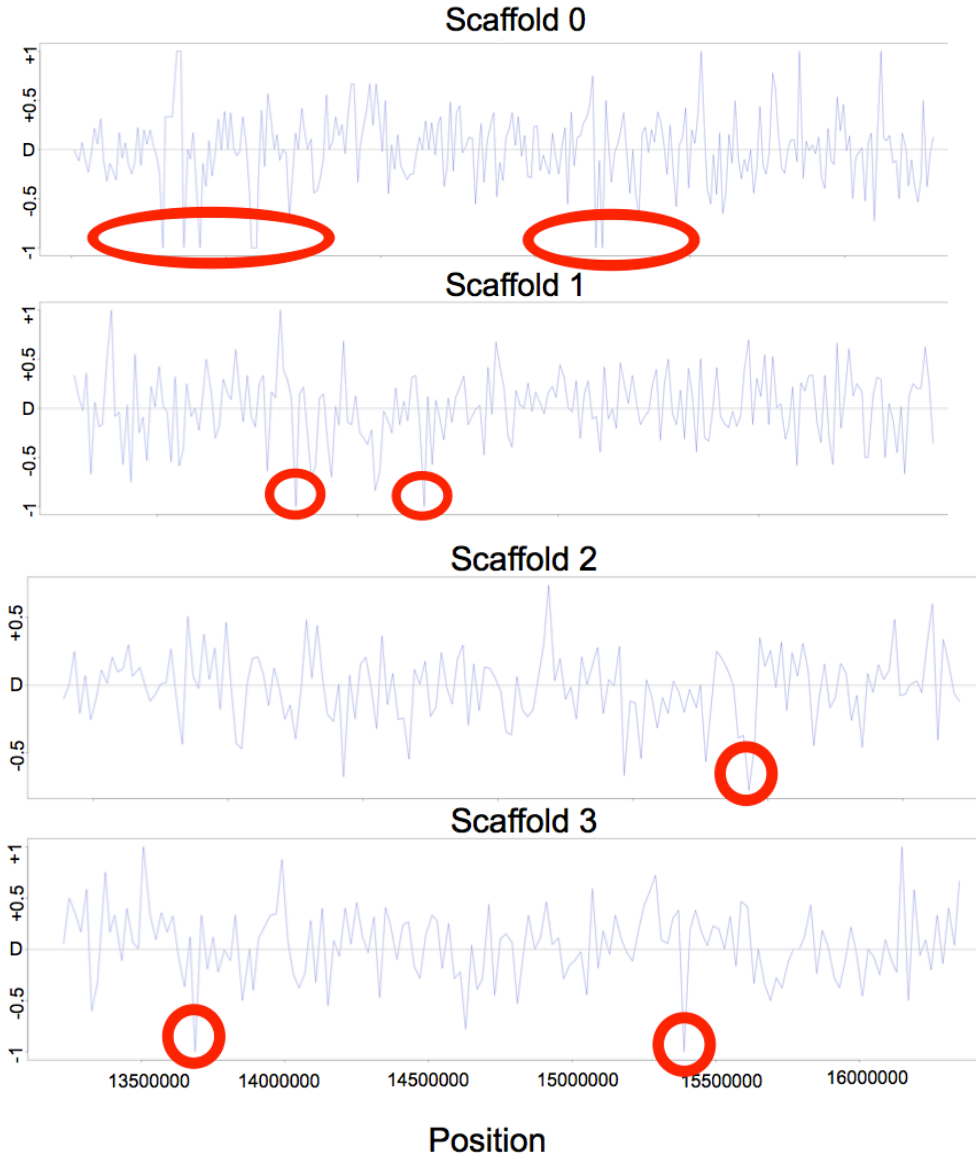
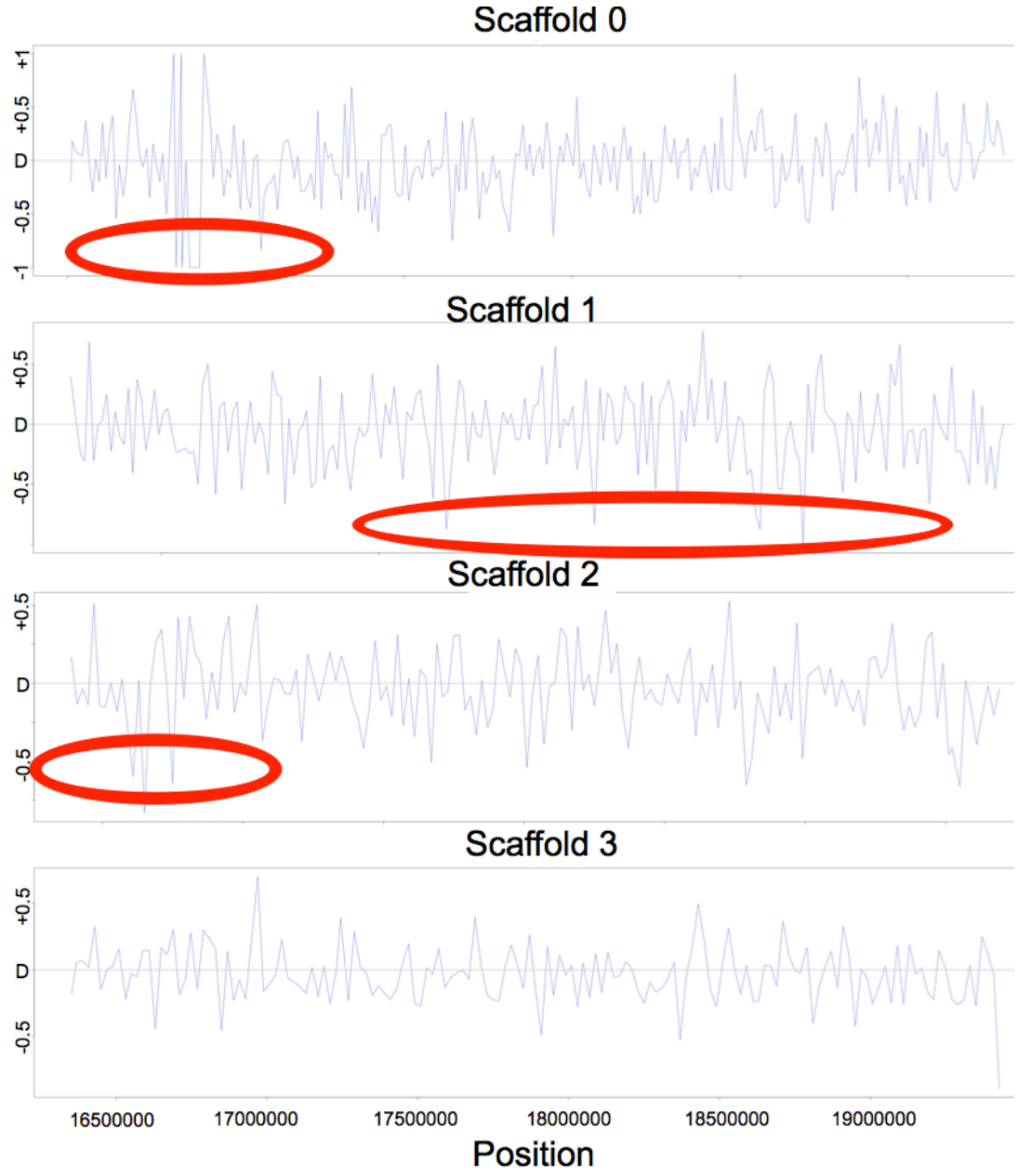


Figure 4. Visualization of D-statistics for introgression patterns for the first four scaffolds of the test as shown in a) Table 1b: P1 – Zürich *S. cynipsea*, P2 – Sörenberg *S. cynipsea*, P3 – Zürich *S. neocynipsea* ; and as shown in b) Table 1d: P1 – Zürich *S. neocynipsea*, P2 – Sörenberg *S. neocynipsea*, P3 – Zürich *S. cynipsea* (right side).

a)



b)


Scaffold 0
+1
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Position

Table 1. Four possible combinations, with three permutations each, of tests for introgression, calculated with Patterson's D-statistics, among sympatric *Sepsis cynipsea* (Cyn) and *S. neocynipsea* (Neo) iso-female lines from Sörenberg and Zürich. Outgroup for all tests was *S. orthocnemis*. Gene flow from P3 to either P1 or P2 is indicated in *italics*. The crucial tests for introgression from one species (P3) to the other (P1 or P2) are in **bold green**. The threshold of significance of a test is Z = 4.

	P1	P2	P3	n ABBA	n BABA	D-statistics	SE	Z
a)	***Zürich Cyn***	**Sörenberg Cyn**	***Sörenberg Neo***	**124739**	**126779**	**-0.008**	**0.002**	**-3.829**
	Sörenberg Cyn	Sörenberg Neo	*Zürich Cyn*	126779	739008	-0.707	0.001	-520.752
	Zürich Cyn	Sörenberg Neo	*Sörenberg Cyn*	124739	739008	-0.711	0.001	-526.692
b)	***Zürich Cyn***	**Sörenberg Cyn**	***Zürich Neo***	**113476**	**115642**	**-0.009**	**0.002**	**-4.266**
	Zürich Neo	*Sörenberg Cyn*	*Zürich Cyn*	698800	115642	0.716	0.001	521.031
	Zürich Cyn	Zürich Neo	*Sörenberg Cyn*	113476	698800	-0.721	0.001	-530.212
c)	***Zürich Neo***	**Sörenberg Neo**	***Sörenberg Cyn***	**130244**	**132242**	**-0.008**	**0.002**	**-3.669**
	Sörenberg Cyn	*Sörenberg Neo*	*Zürich Neo*	591542	132242	0.635	0.001	447.769
	Zürich Neo	Sörenberg Cyn	*Sörenberg Neo*	130244	591542	-0.639	0.001	-449.857
d)	***Zürich Neo***	**Sörenberg Neo**	***Zürich Cyn***	**135188**	**137437**	**-0.008**	**0.002**	**-4.074**
	Zürich Cyn	*Sörenberg Neo*	*Zürich Neo*	603175	137437	0.629	0.001	447.200
	Zürich Neo	Zürich Cyn	*Sörenberg Neo*	135188	603175	-0.634	0.001	-450.503

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APPENDIX

Appendix I

In order to further explore patterns of introgression differentiation, beyond the principal test of iso-female lines reported in the main text, we correspondingly pool-sequenced two Swiss *S. neocynipsea* and four European *S. cynipsea* populations collected in geographic areas of parapatry and sympatry (Table A1). Parapatric *S. cynipsea* populations originated from Dillenburg (Germany) and Pehka (Estonia), in addition to our sympatric populations from the Swiss lowland (Zürich) and the Swiss Alps (Sörenberg) treated in the main document. Sequencing was done using pooled DNA extracted from 20 wild caught males per population. DNA extraction library preparation, genome sequencing and data analysis followed the same methodology described in the main document.

Since pooled population samples exhibit much greater allelic variation within genomes, the program ANGSD (Korneliussen et al., 2014) calculates a consensus genome per population prior to introgression analysis. The results of the introgression tests based population pool-sequences corresponding to Table 1 of the main text are shown in Table A2. ABBA-BABA-statistics indicate significant unidirectional introgression from the Sörenberg *S. neocynipsea* population into the Zürich *S. cynipsea* population (Z = 111.115), supporting our results obtained from the analysis of iso-female lines. Similarly, both parapatric European populations of *S. cynipsea* received genes from the Sörenberg *S. neocynipsea* population (Z = 77.802; Table A2). Presumed directions of gene flow are illustrated in Fig. A1. Interestingly, and in agreement with behavioral patterns of reinforcement detected in our hybridization studies (Giesen et al., 2017 = Chapter 2), no signs of introgression whatsoever were detected between Swiss alpine populations of the species as indicated by the expected 50/50 ratio of ABBA-BABA-patterns and low Z-scores across the genome compared to the other phylogenomic comparisons (Z = -0.450; Table A2). However, as mentioned in the main document these results should be treated cautiously due to the high genetic variation within pooled samples (Futschik & Schlötterer, 2010), making straightforward statistical testing by way of ABBA-BABA-statistics doubtful if not impossible.

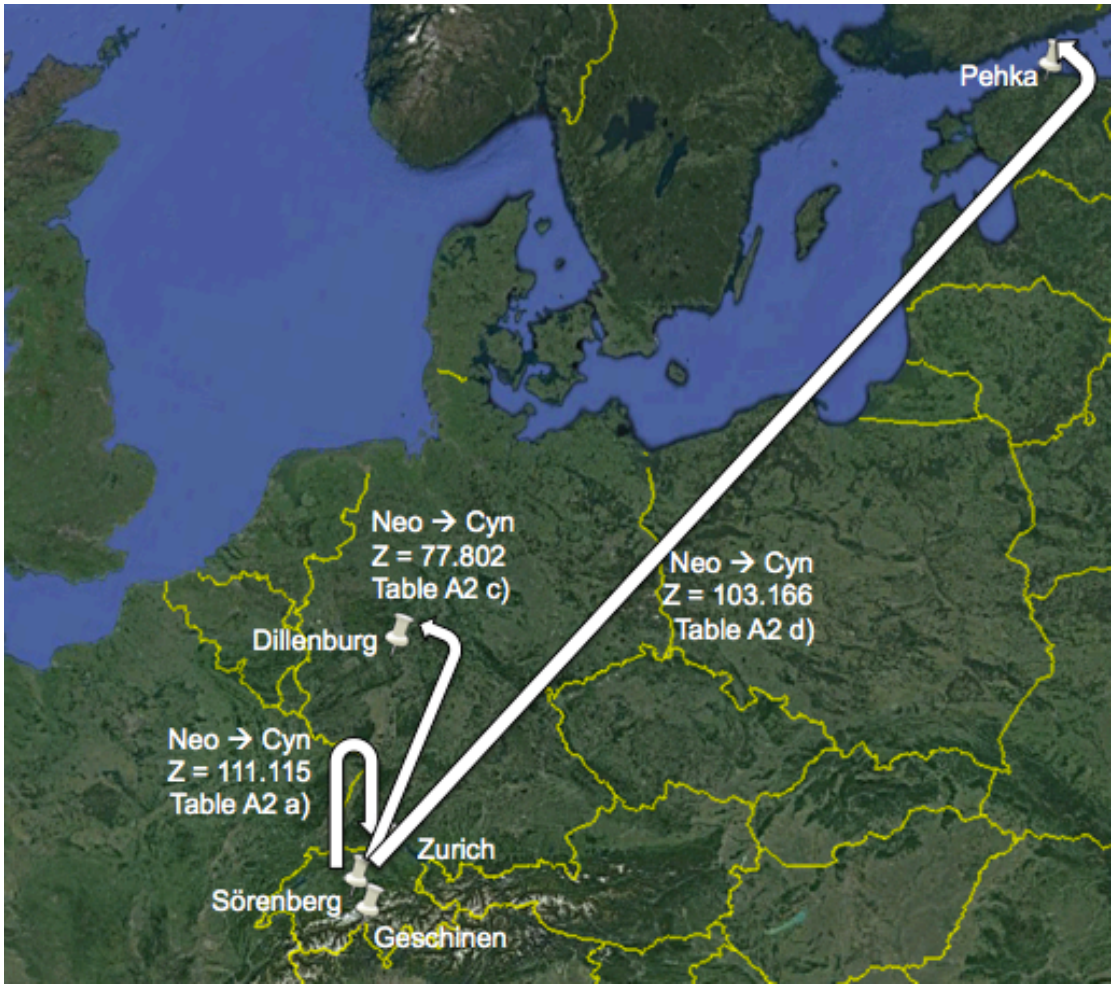
Table A1. Sampling locations and DNA concentrations per sample after DNA extraction and library preparation for a) iso-female lines and b) pooled population sequences.

Species	Population	Country	GPS	DNA concentration [ng/uL] after DNA extraction	DNA concentration [nM] after library preparation	Alignment rate to the outgroup [%]
a)						
S. cynipsea	Sörenberg	Switzerland	46°24'8.24''N 8°21'28.43''E	153.0	0.982	63.48
	Zürich	Switzerland	47°24'0.60''N 8°34'23.97''E	183.0	0.489	65.35
S. neocynipsea	Sörenberg	Switzerland	46°24'8.24''N 8°21'28.43''E	193.0	1.535	63.48
	Zürich	Switzerland	47°24'0.60''N 8°34'23.97''E	210.0	0.513	65.27
b)						
S. cynipsea	Pehka	Estonia	59°28'45.01''N 25°44'52.27''E	86.6	0.457	64.65
	Sörenberg	Switzerland	46°24'8.24''N 8°21'28.43''E	86.3	0.427	65.09
	Dillenburg	Germany	50°32'46.38''N 8°21'44.24''E	81.1	0.267	64.95
	Zürich	Switzerland	47°24'0.60''N 8°34'23.97''E	59.0	0.381	65.81
S neocynipsea	Geschinen	Switzerland	46°49'23.72''N 8°1'54.59''E	116.0	0.296	64.79
	Sörenberg	Switzerland	46°24'8.24''N 8°21'28.43''E	53.4	1.117	65.30

Table A2. Four possible combinations, with three permutations each, of tests for introgression, calculated with Patterson's D-statistics, among European *Sepsis cynipsea* (Cyn) and *S. neocynipsea* (Neo) population pool sequences. Outgroup for all tests was *S. orthocnemis*. Gene flow from P3 to either P1 or P2 is indicated in *italics*. The crucial tests for introgression from one species (P3) to the other (P1 or P2) are in **bold green**. The threshold of significance of a test is Z = 4.

	P1	P2	P3	n ABBA	n BABA	D-statistics	SE	Z
a)	**Sörenberg Cyn**	***Zürich Cyn***	***Sörenberg Neo***	**203499**	**131470**	**0.215**	**0.002**	**111.115**
	Zürich Cyn	Sörenberg Neo	*Sörenberg Cyn*	131470	765416	-0.707	0.001	-528.372
	Sörenberg Cyn	Sörenberg Neo	*Zürich Cyn*	203499	765416	-0.580	0.002	-379.671
b)	**Sörenberg Neo**	**Geschinen Neo**	**Sörenberg Cyn**	**143484**	**143731**	**-0.001**	**0.002**	**-0.450**
	Sörenberg Cyn	*Geschinen Neo*	*Sörenberg Neo*	765869	143731	0.684	0.001	572.644
	Sörenberg Cyn	*Sörenberg Neo*	*Geschinen Neo*	765869	143484	0.684	0.001	572.235
c)	**Sörenberg Cyn**	***Westerwald Cyn***	***Sörenberg Neo***	**155744**	**112062**	**0.163**	**0.002**	**77.802**
	Sörenberg Neo	*Westerwald Cyn*	*Sörenberg Cyn*	678650	112062	0.717	0.001	517.338
	Sörenberg Cyn	Sörenberg Neo	*Westerwald Cyn*	155744	678650	-0.627	0.002	-416.085
d)	**Sörenberg Cyn**	***Estonia Cyn***	***Sörenberg Neo***	**187004**	**123443**	**0.205**	**0.002**	**103.166**
	Sörenberg Neo	*Estonia Cyn*	*Sörenberg Cyn*	740286	123443	0.714	0.001	529.734
	Sörenberg Cyn	Sörenberg Neo	*Estonia Cyn*	187004	740286	-0.597	0.002	-395.104

Figure A1. Map of significant gene flow patterns between European *S. cynipsea* and *S. neocynipsea*.



Appendix II

Script used to visualize D-statistics plots (Fig. 4-6) with R (R Development Core Team, 2011).

```
#################################
# plotDstat.r
# Project: Ahene: ABBA-BABA
# Author: Heidi E.L. Lischer
# Dependencies: -
# Year: 2017
#################################

file <- "/Users/athene/Desktop/PhD/NGS/INTROGRESSIONwriting/Dstats/Table_2b_Dstats.txt"
outfile <- "/Users/athene/Desktop/PhD/NGS/INTROGRESSIONwriting/Dstats/Dstats"

minScafLength <- 1000000
windowWidth <- 40000
windowJump <- 20000

############################


data <- read.table(file, sep="\t", header=TRUE, stringsAsFactors=FALSE)
data <- na.omit(data)
data$pos <- (data$Blockend + data$Blockstart - 1)/2

scafLength <- c()
scafLengthAbs <- c()
data$absPos <- data$pos
add <- 0
scaf <- data$Scaffold[1]
for(row in 1:nrow(data)){
  if(data$Scaffold[row] == scaf){
    data$absPos[row] <- data$absPos[row] + add
  } else{
    scafLength <- c(scafLength, data$Blockend[row-1])
    scafLengthAbs <- c(scafLengthAbs, data$Blockend[row-1] + add)
    scaf <- data$Scaffold[row]
    add <- add + data$Blockend[row-1]
    data$absPos[row] <- data$absPos[row] + add
  }
}
scafLength <- c(scafLength, data$Blockend[nrow(data)])
names(scafLength) <- unique(data$Scaffold)
scafLengthAbs <- c(scafLengthAbs, data$Blockend[nrow(data)] + add)
names(scafLengthAbs) <- unique(data$Scaffold)

dataShort <- data[data$Scaffold %in% names(scafLength[scafLength >= minScafLength]),]

png(paste(outfile, ".png", sep=""), bg="transparent", width=3000, height=800)
plot(dataShort$absPos, dataShort$D_L2, type="l", ylab="D", xlab="position", cex.lab=1.5,
col="blue3",
     main=paste("Scaffolds", ">=", minScafLength), cex.main=2)
abline(h=0, col="gray", lty=1, lwd=2)
for(oneScafLength in scafLengthAbs[scafLength >= minScafLength]){
  abline(v=oneScafLength, col="black", lty=2, lwd=2)
}
dev.off()
```

```
#merge bins
binStart <- 1
binEnd <- windowWidth
dataShortBinned <- data.frame()
while(binEnd <= dataShort$absPos[nrow(dataShort)]){
  oneBin <- dataShort[dataShort$absPos >= binStart & dataShort$absPos <= binEnd, ]
  if(nrow(oneBin) > 0){
    dataShortBinned <- rbind(dataShortBinned, data.frame(Scaffold=oneBin$Scaffold[1],
absPos=mean(oneBin$absPos), D_L2=mean(oneBin$D_L2)))
  }
  binStart <- binStart + windowJump
  binEnd <- binStart + windowJump
}

png(paste(outfile, "_binned.png", sep=""), bg="transparent", width=3000, height=800)
plot(dataShortBinned$absPos, dataShortBinned$D_L2, type="l", ylab="D", xlab="position",
cex.lab=1.5, col="blue3",
     main=paste("Scaffolds", ">=", minScafLength, "binned"), cex.main=2)
abline(h=0, col="gray", lty=1, lwd=2)
for(oneScafLength in scafLengthAbs[scafLength >= minScafLength]){
  abline(v=oneScafLength, col="black", lty=2, lwd=2)
}
dev.off()



#make plot of specific scaffolds ------------------------------------------------
scaffolds <- c("scaffold_0", "scaffold_1", "scaffold_2", "scaffold_3", "scaffold_4")

for(scaffold in scaffolds){
  png(paste(outfile, "_", scaffold, ".png", sep=""), bg="transparent", width=3000, height=800)
  plot(dataShort[dataShort$Scaffold == scaffold, "absPos"], dataShort[dataShort$Scaffold == scaffold,
"D_L2"], type="l",
       ylab="D", xlab="position", main=scaffold, cex.lab=1.5, col="blue3", cex.main=2)
  abline(h=0, col="gray", lty=1, lwd=2)
  dev.off()

  png(paste(outfile, "_", scaffold, "_binned.png", sep=""), bg="transparent", width=3000, height=800)
  plot(dataShortBinned[dataShortBinned$Scaffold == scaffold, "absPos"],
dataShortBinned[dataShortBinned$Scaffold == scaffold, "D_L2"],
       type="l", ylab="D", xlab="position", main=paste(scaffold, "binned"), cex.lab=1.5, col="blue3",
cex.main=2)
  abline(h=0, col="gray", lty=1, lwd=2)
  dev.off()
```

The research presented in this dissertation reiterates the usefulness of conducting extensive research on incipient species considering different types of molecular, behavioral, morphological, and genomic data to study the underlying evolutionary forces of the speciation processes.

In **Chapter 1** we quantified the degree of morphological and molecular differentiation among and within populations of *Sepsis cynipsea* and *S. neocynipsea* from European and North American populations. Molecular analysis of nine highly polymorphic microsatellite markers revealed high divergence between the species indicating that they are indeed distinct (Pont & Meier, 2002). Interestingly, the divergence between continental *S. neocynipsea* was almost of the same magnitude as between the species, signifying ongoing but not yet completed allopatric speciation in a sense of evolution in action. The very low differentiation among populations within species and continents can be explained by large effective population sizes in combination with high amounts of gene flow. While wing morphology mirrored the traditional species concept, presumably driven by stabilizing natural selection, the male armored foreleg was strongly differentiated between species *and* continents, likely driven by sexual selection. The latter is supported by our experimental data showing strong ongoing sexual selection acting on this trait independently of the species composition within habitats (Baur, 2016).

Phylogenetic information (Su, Kutty, & Meier, 2008; Baur *et al.*, 2017, **Chapter 1; Chapter 4**), in combination with obvious differences but also similarities in morphology, behavior, and ecology of *S. cynipsea* and *S. neocynipsea* (Pont & Meier, 2002), lead to the hypothesis that there must be reproductive isolating barriers preventing gene exchange between the two species in areas of co-occurrence. To uncover the mechanisms underlying such putative reproductive isolation we performed forced mating experiments between the species as described in **Chapter 2**. Our results documented female mate choice and species recognition as driving forces in premating isolation with longer copulation latencies as well as low frequency of realized heterospecific relative to conspecific copulations. In these laboratory experiments females showed signs of reinforcement discriminating more strongly against heterospecific partners in pairings of sympatric populations. As F_1 hybrids

showed lower copulation frequencies than conspecific pairings but much higher frequencies than heterospecific pairings, reproductive barriers broke down to some extent with a pattern of hybrid vigor, presumably mediated by the mixture of genes from both species permitting species recognition (Baranwal et al., 2012). Sperm transfer in heterospecific pairings might also be hampered because copulations took longer than in conspecific pairings. **Chapter 3** then documented the consequently expected postmating isolation between the two sister species. Egg-to-offspring viability was significantly reduced, and viable F_1 hybrid offspring showed male sterility but no suppression of female fertility in accordance with Haldane's rule (Haldane, 1922). Ultimately, apparent intrinsic genetic incompatibilities in one hybridization direction (female *S. cynipsea* with male *S. neocynipsea*) prevented further hybridization between the species, while in the opposite direction hybridization was strongly but not ultimately reduced. In contrast, behavioral and reproductive barriers between the continental European and North American *S. neocynipsea* populations were present but not as strong, supporting the traditional view, derived primarily from morphology, that they are indeed the same species (Pont & Meier, 2002), however in the process of incipient speciation based on our microsatellite results in **Chapter 1**.

Ultimately, pre- and postzygotic isolation barriers together can and do prevent gene exchange between the two species in areas of co-occurrence. Although our forced laboratory experiments demonstrated successful hybridization with production of fertile F_1 hybrid females but only sterile hybrid males, this does not necessarily imply that hybridisation also happens in nature. We addressed this question indirectly by estimating the degree of introgression in nature at the genomic level in **Chapter 4**. This study using a phylogenetic ABBA-BABA-approach showed that *S. cynipsea* and *S. neocynipsea* are indeed two genetically distinct species, strengthening our results obtained from **Chapter 1**. However, we also found evidence of bidirectional and unidirectional introgression between the species in sympatric Swiss Alpine and lowland populations that is likely ancient, as suggested by relatively weak signatures of introgression with rather few and small introgressed genetic regions that by now appear homogenized by recombination and/or selection. Therefore, although our laboratory experiments showed that forcing hybridization between the species is possible (**Chapters 2 & 3**), effective means preventing this in nature seem to be at

work in the areas of sympatry in Switzerland studied here, such as strong behavioral species recognition (**Chapter 2**) and/or distinct micro-ecological niches mediating spatio-temporal divergence in reproductive timing. In conclusion, we showed that it is difficult to transfer findings derived from laboratory studies (**Chapter 2 & 3**) directly to natural conditions (see **Chapter 4**), though the former definitely gave us insights into the natural reproductive isolating mechanisms.

Future directions

The work presented here has substantially advanced our understanding of the speciation process in our widespread, closely related sepsid fly species occurring in sym-, para-, and allopatry and, hopefully, in general. This dissertation thus fostered new avenues for future research. Our results showing strong, interacting pre- and postzygotic isolation between the sister species *S. cynipsea* and *S. neocynipsea* uncovered reinforcement, species recognition, female mate choice, and genetic incompatibilities resulting in reduced hybrid viability and hybrid male sterility being at work: evolution in action! However, little is known about the role of heterospecific fertilization after copulation. We here assumed that heterospecific sperm transfer might be disrupted in heterospecific pairings as signified by altered copulation durations, and further that at least in one hybridization direction heterospecific sperm might influence fertilization and egg laying probability. Further work on sperm precedence in hybrid matings would yield insights into sperm-egg-interactions similar to findings by e.g. Palumbi (1999) or Rice (1996).

Crucially, we have merely begun to take advantage of the de-novo whole-genome sequences generated for a total of 5 sepsid species, only two (three) of which were researched in this dissertation. The whole-genome (re-)sequence data generated from our extensive population samples of *S. cynipsea* and *S. neocynipsea*, but also other related sepsid species, will lead to further work in at least three (related) realms. (1) SNP-based population- and phylo-genomic work should strengthen the differentiation results of **Chapter 1**, ultimately helping to uncover the phylogenetic and demographic history of *S. neocynipsea*. Are the North American or the European populations derived (cf. Rohner, Blanckenhorn, & Puniamoorthy, 2016)? (2) Can we find genomic regions involved in latitudinal and/or other geographic adaptation? Based on their distribution differences we hypothesized, and have some preliminary evidence to this effect, that European *S. neocynipsea* are more cold-adapted than

North American *S. neocynipsea*. Finally, (3) numerous genomic SNPs can also serve as markers in planned Quantitative-Trait-Locus (QTL) studies to search for candidate genes mediating the latitudinal or geographic population differentiation in *S. cynipsea, S. neocynipsea, S. thoracica, S. punctum* or *S. orthocnemis*.

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Baur J., Schäfer M.A., Blanckenhorn W.U., & Giesen A. (2017). Patterns of genetic differentiation in sexual and neutral morphological traits among species and populations of dung flies (Diptera: Sepsidae). Chapter 1 of this dissertation.

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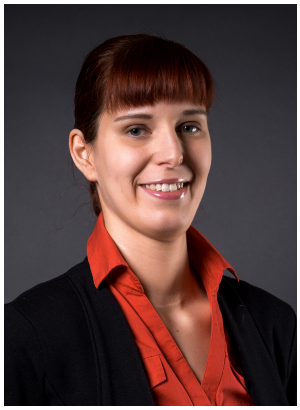

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Certificates	Driver's licence B (2004), Economics-Know-How (will be completed in April 2017), Self-Marketing Skills (2014), Scientific Writing (2014), Project Management (2014), Next Generation Sequencing: assembly, annotation and transcriptomes (2013), Introduction to genome-wide association studies GWAS (2013), How to communicate science to the public (2013), Communication and PR (2009)

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List of Publications

2017 Giesen A., Schäfer M.A., Blanckenhorn W.U. (2017). Behavioural mechanisms of reproductive isolation between two hybridising dung fly species (*Sepsis cynipsea* and *S. neocynipsea*; Diptera: Sepsidae). ANIMAL BEHAVIOUR. Accepted.

2013 Giesen A. (2013). The genetic diversity of the native stone crayfish (*Austropotamobius torrentium*) and effects of habitat fragmentation on the population structure in Canton Zurich, Switzerland. Diploma Thesis, University of Koblenz-Landau.

2011 Giesen A. (2011). Entwicklung von *Daphnia curvirostris*-Populationen über die Zeit: Einfluss der genetischen Diversität auf die Populationsstruktur. Case Study, University of Koblenz-Landau.

Contributions to international Meetings

2017 *Patterns of postzygotic isolation reveal possible hybridization between two closely related dung fly species in nature*
biology17, Bern (CH), 02.-03.02.2017, Poster

2016 *Mechanisms of pre- and postzygotic isolation between two closely related dung fly species Sepsis cynipsea and S. neocynipsea (Diptera: Sepsidae)*
Ecology and Behaviour 2016, Lyon (FRA), 27.06.-01.07.2016, Poster
High inter-specific and inter-continental genetic differentiation between populations of the sister species Sepsis cynipsea and S. neocynipsea (Diptera: Sepsidae)
mind the gap 2016, Vienna (AT), 31.10.-01.11.2016, Poster

2015 *The evolution of reproductive isolation between two hybridizing dung fly species Sepsis cynipsea and S. neocynipsea (Diptera: Sepsidae)*
eseb2015, Lausanne (CH), 09.-14.08.2015, Poster
The evolution of reproductive isolation between two hybridizing dung fly species
Special Seminar University of Bonn (D), 28.05.2015, Talk

2014 *The evolution of reproductive isolation between the two hybridizing dung flies Sepsis cynipsea and S. neocynipsea (Diptera: Sepsidae)*
SGE2014, Ascona (CH), 18.-22.05.2014, Poster

2013 *The genetic diversity of the native stone crayfish (Austropotamobius torrentium) and effects of habitat fragmentation on the population structure in Canton Zurich, Switzerland*
PACE13, Basel (CH), 06.02.2013, Talk